NUMBER 2

L

OLOGY

TY OF AMERICAN

# PRELIMINARY REPORT ON SYNTHETIC MEDIA[1]

C. J. T. DORYLAND

*North Dakota Experiment Station, Fargo, North Dakota*

## THEORETICAL DISCUSSION

By a "Synthetic Medium" is meant a solution which contains only compounds of known composition and structure. Any medium which includes compounds of unknown composition or structure is not a synthetic medium. This paper deals both with synthetic nutrient solutions, and, with solid synthetic media formed by the precipitation of an agglutinant from compounds of known composition and structure. In the latter case, the agglutinant formed by precipitation from known compounds should have a known structure and composition. We should properly exclude from the list of synthetic media all those which contain substances of unknown composition or structure, such as meat extracts, proteins of unknown structure, agar and gelatine. In order, however, to illustrate the present conception of possible media, the scheme presented includes many compounds of unknown composition and structure, such as the polysaccharids, tannins, glucosides and proteins. These compounds which have either an unknown composition or unknown structure are placed in their logical position in the systematic arrangement, because their decomposition products are more or less known and because in nature they, or their decomposition products, furnish the principal source of energy to saprophytic microorganisms, and because it may be necessary to fall back upon some of them in order to secure media for such microorganisms as cannot utilize media made of simpler compounds. However, media which contain such compounds of unknown composition or structure cannot properly be classed as synthetic.

[1] Presented at Seventeenth Annual meeting of the Society of American Bacteriologists, Urbana, Illinois, December 28, 1915.

It is clear from the results of numerous investigators that microorganisms can utilize various carbohydrates, alcohols and salts of fatty acids as sources of carbons, both for energy material and cell construction. It has, also, long been known that the optical isomers of a substance containing an asymmetrical carbon atom behave very differently toward biological agents, such as yeast, molds, and bacteria. The classical researches of Pasteur showed that *Penicillium glaucum*, assimilated only d-tartaric acid and not the l-tartaric acid. Recent investigations have shown, however, that this organism will also decompose the l-tartaric but less rapidly than the d-tartaric form. Likewise there are other organisms, bacteria, yeast and molds, some of which prefer a d-form of an isomeric compound while others prefer the l-form of the isomer. Investigations of the action of yeast on all the known hexoses has shown that only four are fermented, viz., the d-forms of glucose, mannose, galactose and fructose. When the behavior of different species of yeast toward these hexoses is studied, it is found without a single exception that any species of yeast which ferments any one of the three hexoses, glucose, mannose or fructose, likewise ferments all three of them. We know, too, from the work of E. Fischer and his associates that certain enzymes which are able to decompose certain organic compounds cannot attack their opposite isomers. Thus, for example, $\alpha$-methyl glucoside is hydrolyzed by the maltase ($\alpha$-glucosidase) of yeast, while $\beta$-methyl glucoside is hydrolyzed by emulsin ($\beta$-glucosidase). Emulsin does not act on the $\alpha$-glucoside and maltase has no effect on the $\beta$-glucoside. So fundamental is this action that the composition of an organic compound may often be determined by its behavior in the presence of an enzyme. While lack of knowledge concerning the behavior of optically inactive compounds toward enzymes does not permit us to speak as definitely as we may concerning the optically active, nevertheless it is probable that an enyzme is capable of attacking only a certain group, or formation. Such, for example, is the action of myrosin upon sinigrin and other sulphur containing glucosides, where the change is confined to the sulphur linkage. This

property of the enzyme no doubt regulates the ability of the parent cell to utilize a compound, consequently, organisms can utilize as a source of energy and carbon for cell construction only those compounds whose configuration, or the configuration of a radical therein, matches the configuration of their enzymes. By commencing with the simplest carbon compounds capable of yielding energy we may, by admitting only one energy material at a time build up a series of synthetic media which are mutually inclusive and exclusive. That is, those organisms which have an enzyme in common can utilize a given compound, while those which do not possess the necessary enzyme will not develop, providing the compound is not too complex; otherwise, some of the decomposition products may have a suitable configuration. We find that carbon compounds having a certain configuration or certain atomic groups can be utilized by all those microorganisms which develop the requisite enzyme. Therefore, as far as energy is concerned, it is possible to prepare a medium which will allow the growth of only a given number of organisms.

Numerous investigators have shown that bacteria as well as molds can utilize ammonia nitrogen. Gerlach and Vogel isolated and studied several forms of bacteria which were capable of utilizing nitrate nitrogen. It is a well known fact that a large number of molds also readily assimilate nitrate nitrogen. Renato Perrotti pointed out the fact that certain bacteria were capable of utilizing cyanide nitrogen. The assimilation of either ammonia, nitrate or cyanide nitrogen was dependent, always, upon the presence of suitable energy materials.

Previous work indicates that the other nutrient elements necessary, for bacteria at least, are probably common to all, therefore, the development of synthetic media must follow two lines of cleavage—first, according to carbon compounds required for energy, and second, according to nitrogen compounds required for nutrition.

In order to make the résumé complete we need only mention those bacteria which require a source of carbon for energy and utilize free nitrogen, those which obtain their energy by the

oxidation of ammonia or nitrite nitrogen, those bacteria which obtain their energy by the oxidation of sulphur and those which obtain their energy from the oxidation of iron carbonate. We are now in a position to summarize the food requirements of microorganisms. They need in general the common nutrient salts. Their nitrogen requirements differ, some being able to utilize ammonia, some nitrate, some cyanide and probably some nitrite nitrogen. They differ too in the compounds which they use as sources of energy. Among the prototrophic forms we find, respectively, those groups which can utilize iron, sulphur, ammonia, nitrite or a non-nitrogenous organic compound as a source of energy, while among the saprophytic and parasitic forms we find those which can utilize the non-nitrogenous organic compounds as sources of energy, as well as those which can utilize nitrogenous organic compounds. If we prepare nutrient synthetic solutions containing one of the simplest nitrogen and one of the simplest carbon compounds there will grow in that medium only those organisms which can utilize both the nitrogen and carbon compounds present. For example, a medium which contains the necessary nutrient mineral salts and ammonia as a source of nitrogen. with a formate as a source of energy will allow the development of only those organisms which can assimilate ammonia nitrogen and utilize a formate as a source of energy, and for the carbon of its cell construction. If an organism is present which can assimilate ammonia nitrogen but cannot utilize a formate as a source of energy and cell construction it cannot develop. Conversely, if an organism is present which can utilize the formate but not the ammonia nitrogen there will be no growth. Therefore, starting with the simplest nitrogen and carbon compounds we can arrange a series of media having a common source of nitrogen and a changing source of energy, or *vice versa*, having a common source of energy but a changing source of nitrogen. We have, therefore, the possibility of developing as many media as the product of the number of nitrogen sources multiplied by the number of nitrogen free energy compounds. To these may be added a large number of media which may be made from

compounds containing both energy and nitrogen. For such organisms as are not able to develop upon a medium containing one of the simplest nitrogen salts, and a non-nitrogenous compound as a source of energy, we may develop a suitable medium by using a solution containing the essential mineral salts to different portions of which we add a different nitrogenous compound until we find a nitrogenous compound upon which it will develop. However, we must exercise caution here, particularly in the employment of complex compounds for mixed floras, because as a general rule the more complex the compound the greater the number of species which can utilize the compound or its degradation products. It is by simplicity of compounds that we may hope to control the growth of the great mass of saprophytes.

Possible media may be grouped according to their energy requirements as follows:

THE AMMONIA GROUP

Ammonia as a source of energy, no other form of nitrogen.

THE NITRITE GROUP

Nitrite as a source of energy, no other form of nitrogen.

THE SULPHUR GROUP

Sulphur as a source of energy, + ammonia.

THE IRON GROUP

Iron as a source of energy, + ammonia.

THE CYANIDE GROUP

Cyanide as a source of energy (no other form of nitrogen).
Cyanide as a source of energy, + ammonia nitrogen.

NITROGEN FIXING GROUP (Oligonitrophilic)

Compounds of carbon with hydrogen and oxygen as sources of energy, no nitrogen added.

THE ALIPHATIC ACID GROUP

The Aliphatic acids as a source of energy, + ammonia nitrogen.

| *Monobasic* | *Monobasic hydroxy* | *Monobasic ketonic* |
|---|---|---|
| Formic | Glycolic | Pyruvic |
| Acetic | d-lactic | Acetoacetic |
| Propionic | l-lactic | Levulinic |
| Butyric | Hydracrylic | |
| Isobutyric | | |
| Isovaleric | | |
| Normal Valeric | | |

| *Di-basic* | *Hydroxy di-basic* | *Unsaturated di-basic* |
|---|---|---|
| Oxalic | Malic | Fumaric |
| Malonic | Tartaric (dextro) | Maleic |
| Succinic | Tartaric (levo) | Mesaconic |
| Iso Succinic (Methyl malonic) | Meso-Tartaric | Citraconic |

*Tri-basic*
Citric
Aconitic
Tricarballylic

THE AROMATIC ACID GROUP

Aromatic acids as a source of energy, + ammonia nitrogen.

| | |
|---|---|
| Benzoic | Salicylic |
| Anisic | Gallic |
| Tannic | Quininic |
| Phthalic | Amygdalic |
| Phenylacetic | Mandelic |
| Cinnamic | |

THE ALDEHYDE GROUP

Aldehydes as a source of energy, + ammonia nitrogen.

| | |
|---|---|
| Formaldehyde | Benzoic aldehyde |
| Acetic aldehyde | Cuminol |
| Propyl aldehyde | Cinnamic aldehyde |
| Butyl aldehyde | Salicylaldehyde |
| Butyl aldehyde, iso | Vanillin |
| | Piperonal |

THE KETONE GROUP

Ketones as a source of energy, + ammonia nitrogen.

Aceton
Butanon
Pentanon

THE ALCOHOL GROUP

Alcohols as a source of energy, + ammonia nitrogen.

*Monohydric alcohols*
Methyl alcohol
Ethyl alcohol
Propyl alcohol, normal
Propyl alcohol, iso
Butyl alcohol, iso
Butyl alcohol, primary normal
Butyl alcohol, secondary normal
Amyl alcohol, iso primary
Amyl alcohol, active

| *Polyhydric alcohols* | *Aromatic alcohols* |
|---|---|
| Ethylene glycol | Benzyl alcohol |
| Glycerol | |
| Erythritol | |
| Arabitol | |
| Xylitol | |
| Mannitol | |
| Dulcitol | |
| Sorbitol | |
| Persitol | |
| Volemitol | |
| Adonitol | |

THE CARBOHYDRATE GROUP

Carbohydrates as a source of energy + ammonia nitrogen.

*Monosaccharids*

| *Trioses* | *Tetroses* | *Pentoses* |
|---|---|---|
| Glyceric aldehyde | d and l-Erythrose | d and l-Arabinose |
| Dioxyacetone | d and l-Threose | d and l-Xylose |
| | | l-Ribose |
| | | l-Lyxose |

*Hexoses*

| *Methyl Pentoses* | *Mannitol series* |
|---|---|
| Rhamnose | |
| Fucose (Rhodeose) | d and l-Glucose |
| Chinovose | d and l-Mannose |
| | Fructose |
| | Sorbose |
| | *Dulcitol series* |
| | d and l-Galactose |
| | d and l-Talose |
| | Tagatose |

| *Heptoses* | *Octoses* | *Nonoses* |
|---|---|---|
| Mannoheptose | Mannoctose | Mannononose |
| Glucoheptose | Glucooctose | Gluconononose |
| Galacto heptose | Galactoose | |

| *Disaccharids* | *Trisaccharids* | *Tetrasaccharid* | *Polysaccharids* |
|---|---|---|---|
| Maltose | Mannotriose | Stachyose | Dextrins |
| Iso maltose | Rhamninose | | Soluble starches |
| Gentiobiose | Raffinose | | Starches, Inulin, etc. |
| Cellobiose | Gentianose | | Gums |
| Lactose | Melicitose | | |
| Iso lactose | | | |
| Melibiose | | | |
| Turanose | | | |
| Sucrose | | | |
| Trehalose | | | |

THE NON-NITROGENOUS GLUCOSIDE GROUP

Non-nitrogenous Glucosides as a source of energy, + ammonia nitrogen.

*Phenolic glucosides*
- Arbutin
- Methylarbutin
- Phlorhizin
- Glycyphyllin
- Hesperidin
- Naringin
- Iridin
- Baptisin

*Alcoholic glucosides*
- Salicin
- Populin
- Coniferin
- Syringin

*Aldehydic glucosides*
- Helicin
- Salinigrin

*Acidic glucosides*
- Convolvulin
- Jalapin
- Strophantin
- Gaultherin

*Oxycumarin glucosides*
- Aesculin
- Daphnin
- Fraxin

*Oxyanthraqunone glucosides*
- Ruberythrinic acid
- Rubiadin glucoside
- Frangulin

*Oxyflavone glucosides*
- Apiin
- Fustin
- Quercitrin
- Sophorin
- Xanthorhamnin

*Miscellaneous glucosides*
- Saponins
- Digitonin
- Digitalin
- Saponarin
- Camatambin

THE ESTER GROUP

Esters[2] as a source of energy, +ammonia nitrogen.

The entire series, after the first few types, may be repeated three times by substituting in place of the ammonia first, nitrite, -second, nitrate, -third, cyanide nitrogen. We would have then, nitrite-aliphatic-acid group; nitrite carbohydrate group; etc., or the nitrite series; and the nitrate aliphatic acid group and nitrate alcohol group, etc., or the nitrate series and cyanide-aliphatic-acid group and cyanide aldehyde group, etc., or the cyanide series.

Should occasion arise we may exclude all forms of nitrogen and carbon from the solution and substitute a series of amino-acids, amides, amino compounds, ureides, proteins, nitrogenous, glucosides, or cyanogen compounds, and thus build up series of media with each respective group.

[2] The utility of this group is doubtful, it being probable that if one of the constituents of the ester is available to an organism the ester may be also.

## PREPARATION OF MEDIA

### *Conditions*

All water used was double distilled from glass retorts and condensers. All chemicals used were the purest the market afforded. Each chemical was tested for impurities before use. All measures of growth were macroscopic.

Tests with liquid solutions were made in test tubes. Tests with solid media were made in Petri dishes. The incubator temperature adopted was 28°C. All media used were made neutral to phenolphthalein. The influence of sterilization by heat on composition and structure may be eliminated in most instances where an acid is used as a source of energy by preparing a medium in such a manner that it automatically sterilizes itself. This will be explained under preparation of media. Whenever this has not been possible the compound whose structure is liable to be influenced by high temperatures, has been sterilized by itself in neutral aqueous solution and added to the other sterile constituents, in correct proportion, by means of a sterile pipette. All transfers were made from cultures 48 to 96 hours old grown on standard agar.

### *Solid synthetic media*

It will be necessary to describe the solid media first in order to explain the reason for the concentration of the liquid media. After numerous trials with starch, cellulose, aluminum hydroxide, iron hydroxide and washed agar as agglutinants, it was finally demonstrated that silica jelly was the most suitable solid medium. The silica jelly was made by a modified "Stevens Temple Method." (Centbl. Bakt., etc., II abt., vol. 21, 1908, p. 84.)

The method consisted essentially of dissolving c. p. $K_2SiO_3$ and c.p. $Na_2SiO_3$ in water in sufficient amounts to give a concentration of 34.2732 grams of $H_2SiO_3$ per liter. One half this concentration of $H_2SiO_3$ per liter gives a medium which will solidify in approximately five minutes, thus making a medium suitable for plating. The mixture of sodium and potassium sili-

cate gives us sodium and potassium salts in the final medium instead of only sodium salts. thereby lessening the danger of too great a concentration of sodium salt.

The detrimental influence of too great a concentration of the sodium and potassium salts can be still further lessened by using a mixture of acids; for example, we may use equivalent solutions of HCl, $H_2SO_4$ and $H_3PO_4$, thus giving in the finished medium chlorides, sulphates and phosphates of both sodium and potassium. Experiment demonstrated that the "Stevens Temple Method" might be still further modified by eliminating the $MgCO_3$ or $Na_2CO_3$.

The time of precipitation and gelatinization of $H_2SiO_3$ depends largely on two factors, first, reaction and second, concentration. Gelatinization is delayed or entirely prevented by either an excess of acid or an excess of the $Na_2SiO_3$ or $K_2SiO_3$. The most rapid gelatinization with any concentration takes place in a neutral solution.

The solid synthetic media were prepared in the following manner: Solutions of HCl, $H_2SO_4$ and $H_3PO_4$ were each standardized separately against the $Na_2SiO_3$ and $K_2SiO_3$ solution, so that 1 cc. of each acid would just neutralize 1 cc. of the silicate solution. Whenever an organic acid such as formic, acetic, lactic or tannic, etc., was used as a source of energy it was made in sufficient concentration to just neutralize an equal volume of the silicate solution. These acids, that is the HCl, $H_2SO_4$, $H_3PO_4$, and the organic acid (let us say acetic) were then mixed together in such proportion that the resulting salts from the sodium and potassium silicate would be present in the final silicate medium in quantities, approximately, inversely proportional to their osmotic action, thus giving a minimum osmotic pressure. Before standardizing the HCl there was added to it 0.5 gram $MgSO_4$, 0.01 gram $CaCO_3$ or CaO, 0.01 gram of $Fe_2(SO_4)_3$ and 0.01 gram of Mn $SO_4$, per liter. Ammonia nitrogen was added to the HCl as ammonium sulphate. Cyanide nitrogen was added to the HCl as potassium ferricyanide. Nitrite nitrogen was added to the neutral solution in the Petri dish, nitrate nitrogen was added to the acid mixture

as $HNO_3$. All the sources of nitrogen[3] were added in proportion to give one gram of their respective salts per liter. The mixture of acids was then placed in a sterile flask plugged with cotton and the flask connected with an automatic burette so that the burette would fill by siphon. The silicate solution was placed in another sterile cotton plugged flask and connected with another automatic burette, so that it too would fill by siphon. Each burette was allowed to fill and then stand several hours before use, so as to sterilize completely the flasks and burettes. The overflow cup of the burette was plugged with cotton, to prevent contamination from the air during titration. When sterile, 5 cc. of the acid mixture was added to a sterile Petri dish, after which there was added 5 cc. of the silicate solution. The plate was then rotated to mix the two solutions thoroughly, and then inoculated. If a non-acid compound was used as a source of energy (say glucose) a sufficient amount of a sterile aqueous solution, to give 10 grams per liter, was added at this point. Numerous tests proved both the acid and silicate solutions to be sterile in less than one hour. The resulting silica medium was neutral to phenolphthalein and set firmly in approximately five minutes. When a compound other than an acid was used as a source of energy it was added from a sterile aqueous solution to the medium in the Petri dish just before inoculation. The growth of organisms upon this solid medium resulted generally in quite typical colonies, although with a few organisms somewhat peculiar developments took place.

The liquid media used had a concentration similar to the above with the exception that $H_2SiO_3$ was absent. It is possible that some organisms which failed to grow might have grown in a lesser concentration. This was not determined because the first consideration was to develop solid synthetic media, and a more dilute silicate solution would not solidify soon enough; therefore the liquid media used as a check for the solid media must necessarily be of the same concentration. Parenthetically, it may be stated that the results of the liquid

[3] In future experiments, the availability of different salts of ammonia, nitrite, nitrate and cyanid must be tested.

and solid media did not always agree. The acetic ammonium medium will give an approximate idea of the composition and concentration.

*Acetic-ammonia-silicate medium*

| | |
|---|---|
| $H_2SiO_3$ | 17.1366 |
| NaCl | 1.1620 |
| $Na_2SO_4$ | 2.8209 |
| $NaC_2H_3O_2$ | 1.6343 |
| $Na_2H\ PO_4$ | 4.2330 |
| K Cl | 3.2954 |
| $K_2SO_4$ | 7.7024 |
| $K\ C_2H_3O_2$ | 4.3357 |
| $K_2H\ PO_4$ | 11.5500 |
| $MgSO_4$ | 0.5000 |
| CaO | 0.0100 |
| $Fe_2(SO_4)_3$ | 0.0100 |
| MnSO | 0.0100 |
| $(NH_4)_2SO_4$ | 1.0000 |
| Water double dist | 1000.0000 |

The acetic ammonia solution agrees with the above, excepting that it does not contain the $H_2SiO_3$, It may be made as follows: Make a solution of KOH and one of NaOH so that 1 cc. of each alkali will just neutralize 1 cc. of one of the acids which was standardized against the silicate solution. The KOH and NaOH solutions are then mixed in such proportions that when the mixture is brought in contact with the acid mixture the resulting sodium and potassium chlorides, sulphates, phosphates and acetates are present in quantities inversely proportional to their osmotic action. The acids may be prepared as previously described. The alkali mixture and the acid mixture may be placed in their respective bottles and connected by siphon with automatic burettes. Allow the burettes to fill as with the silicate medium and when sterile equal quantities of the alkali mixture and of the acid mixture may be run from the burettes in a suitable vessel.

It must be borne in mind that when glucose or an alcohol, or any other non-acid compound is used as a source of energy the proportion of each of the remaining salts increases. Likewise, the relative proportion changes when another acid, such as formic or lactic, is used in place of acetic acid. This fluctua-

tion of the chloride, sulphate and phosphate content of the medium might be eliminated in some instances[4] by adding the organic acids in such quantities that the resulting organic salt of sodium and of potassium should always contain equivalent quantities of these bases. Such a procedure might, however, give greater fluctuations in the osmotic action than the former procedure. This question, with many others, must be left for future investigators.

For convenience there is given below a concise method for making the ammonia-acetate-silicate medium and the ammonium acetate solution. These figures give quantities of salts which are only approximately inversely proportional to their osmotic action. Up to the present time the writer has been unable to secure all the necessary ionization constants.

Weigh out: 8.40 grams of c.p. $Na_2SiO_3$, 24.00 grams of c.p. $K_2SiO_3$ and dissolve in 500 cc. of distilled water. Dilute HCl to a concentration so that 1 cc. of the silicate solutions does not quite neutralize 1 cc. of the HCl. Add to the HCl 0.5 gram of $MgSO_4$, 0.01 gram of CaO, 0.01 gram of $Fe_2SO_4$ 0.01 gram of $MgSO_4$ and 1 gram of $(NH_4)_2\ SO_4$, and standardize the resulting HCl solutions against the silicate, using methyl orange as indicator, so that 1 cc. is equivalent to 1 cc. of the silicate solution.

Standardize a solution of $H_2SO_4$ in the same way, omitting the salts.

Standardize $H_3PO_4$ and $CH_3COOH$ in a similar manner omitting the salts and using phenolphthalein as indicator.

The acids may then be mixed in the following proportion:

| | cc. |
|---|---|
| HCl | 153.5 |
| $CH_3COOH$ | 153.5 |
| $H_2SO_4$ | 77.0 |
| $H_3PO_4$ | 116.0 |

One cubic centimeter of this acid mixture will just neutralize 1 cc. of the silicate mixture, using phenolphthalein.

[4] Acids whose solubility in water is so low that solutions, equivalent to the silicate solution, cannot be obtained will still further complicate the question.

*Ammonia-acetate solution*

Prepare a N/0.2578 solution of NaOH and a N/0.6205 solution of KOH. Mix these solutions in equal proportions and substitute in the place of the silicate solution. The procedure from here on is the same as for the silicate medium.

*Results with pure cultures*

It has been the object in working with these media to test as many pure cultures of microorganisms upon each medium as could be obtained. In this way we shall be able eventually to determine all the compounds which each organism can use as a source of energy as well as those from which it can obtain its nitrogen. This will eventually enable us to group the known organisms (yeast and molds as well as bacteria) according to their ability to utilize energy material of a given chemical structure and configuration. The same thing applies to their nitrogen requirements. When this has been accomplished we may then eliminate all media suggested in the above groups that are common to a given group of organisms and retain for practical use the one which is the most serviceable. Thus eventually the above group of synthetic media will be reduced for general use to a few which are mutually inclusive and exclusive with occasional employment of others for special studies.

Several of the media described above have been tested upon 225 pure cultures of bacteria, 50 cultures of molds and 6 cultures of yeast. In the following table will be found the positive results obtained from three glucose media and three acetate media; only positive results are given because of lack of space.

Out of 225 pure cultures of bacteria tested the following results were obtained: On the glucose-ammonia medium there were 83 positive growths; on the glucose-nitrate medium 70 positive growths; on the acetate-ammonia medium 25 positive growths; and on the acetate-nitrate medium 17 positive growths. With the glucose-cyanide medium there were 7 positive growths; and with the acetate-cyanide medium there were 2 positive growths.

*Positive growths on glucose ammonia, glucose nitrate, glucose cyanide and acetate ammonia, acetate nitrate and acetate cyanide media*

| NUMBER OF ORGANISM | GLUCOSE | | | ACETATE | | |
|---|---|---|---|---|---|---|
| | $NH_3$ | $NO_3$ | CN | $NH_3$ | $NO_3$ | CN |
| 7 | | X | | | | |
| 10 | | | | | | |
| 15 | | X | | | | |
| 17 | | X | | | | |
| 18 | X | X | | | | |
| 22 | X | X | | X | | |
| 23 | X | X | | X | | |
| 29 | X | X | | X | X | |
| 30 | X | X | | | X | |
| 37 | X | | | | | |
| 40 | | X | X | | | |
| 47 | X | X | | X | X | |
| 52 | X | X | | X | | |
| 56 | | | | | | |
| 59 | | | X | X | | X |
| 72 | | X | | | | |
| 80 | | X | | | | |
| 88 | X | X | | X | | |
| 103 | X | X | | X | | |
| 110 | | X | | | | |
| 112 | X | X | | X | | |
| 116 | | X | | X | | |
| 121 | | X | | | | |
| 122 | | X | | X | | |
| 123 | | X | | X | | |
| 124 | X | X | | | | |
| 125 | X | X | | X | | |
| 126 | X | X | | | | |
| 130 | X | | | | | |
| 135 | X | X | | X | | |
| 136 | X | X | | | | |
| 137 | X | X | | | X | |
| 141 | X | | | | | |
| 142 | X | | | | X | |
| 143 | | X | X | | | |
| 144 | X | | | | | |
| 152 | X | X | | X | X | |
| 161 | X | X | | X | | |
| 170 | X | X | | X | | |
| 175 | X | X | | | | |
| 183 | | X | | X | | |

| NUMBER OF ORGANISM | GLUCOSE | | | ACETATE | | |
|---|---|---|---|---|---|---|
| | $NH_3$ | $NO_3$ | CN | $NH_3$ | $NO_3$ | CN |
| 184 | X | | X | X | | |
| 185 | X | X | | | | |
| 187 | X | X | | X | | X |
| 193 | | X | | | | |
| 195 | X | X | | | | |
| 197 | | X | | | | |
| 224 | | X | | | | |
| 229 | X | | | | | |
| 230 | | X | | | | |
| 232 | X | X | | | | |
| 235 | X | | | | | |
| 239 | X | X | | | | |
| 240 | X | X | | | | |
| 242 | X | X | | | | |
| 244 | | X | | | | |
| 246 | X | | | | | |
| 257 | X | X | | | | |
| 277 | X | | | | | |
| 282 | X | | | | | |
| 304 | X | X | | | | |
| 306 | X | X | | | | |
| 318 | | X | | | | |
| 242 | X | | | | | |
| 347 | | X | | | | |
| 352 | X | | | | | |
| 370 | X | X | | | | |
| 371 | X | | | | | |
| 373 | X | | | | | |
| 374 | X | | | | | |
| 378 | | X | | | | |
| 380 | | X | | | | |
| 383 | X | | | | | |
| 385 | | X | | | | |
| 405 | | X | | | | |
| 424 | X | X | | | | |
| 429 | X | X | | | X | |
| 436 | X | X | | | | |
| 439 | | X | | | | |
| 468 | X | | X | | | |
| 475 | | X | | | | |
| 477 | X | | | | | |
| 478 | X | | | | | |
| 487 | X | | | | | |
| 491 | | X | | | | |

| NUMBER OF ORGANISM | GLUCOSE | | | ACETATE | | |
|---|---|---|---|---|---|---|
| | $NH_3$ | $NO_3$ | CN | $NH_3$ | $NO_3$ | CN |
| 492 | X | | | | | |
| 495 | X | | | | | |
| 497 | X | | | | | |
| 505 | X | | | | | |
| 506 | | X | | | | |
| 527 | X | X | | | | |
| 528 | X | | | | | |
| 539 | X | | | | | |
| 540 | X | | | | | |
| 541 | X | | | | | |
| 544 | X | | | | | |
| 549 | X | X | | | | |
| 555 | X | X | | | | |
| 572 | X | X | | | | |
| 573 | | X | | | | |
| 574 | X | | | | | |
| 575 | X | | | | | |
| 579 | X | | | | | |
| 583 | X | X | | | | |
| 589 | X | X | | | | |
| 592 | X | | | | | |
| 593 | X | X | | | | |
| 594 | | | | | | |
| 595 | X | | | | | |
| 596 | X | | | | | |
| 598 | X | | | | | |
| 601 | X | | | | | |
| 602 | | | | | X | |
| 603 | | | | | | |
| 610 | | | | | | |
| 612 | | | | | X | |
| 614 | | | | | X | |
| 615 | | | | | | |
| 617 | | X | | | | |
| 618 | | | | | X | |

Laboratory numbers have been used purposely because the writer is not positive concerning the identity of some of the organisms used in the test. It must be borne in mind that the above results would be even more striking if the negative growths were included.

Space does not permit the presentation of more data, but the

above table demonstrates clearly the possibilities of the proposed synthetic media. It is apparent at once that the pure species of bacteria included in these experiments may be grown on some of the above synthetic media and controlled, to a certain extent, at least, by changing the energy or nitrogen source.

## SUMMARY

The above scheme differs radically from the attempts to secure a universal medium, i.e., one upon which most bacteria of the saprophytic or parasitic groups will grow. It is on the contrary an attempt, by the use of definite sources of energy and definite sources of nitrogen, to exclude all species but those which can use the particular source of energy and nitrogen included in each case. If we are thus able to devise a series of mutually inclusive and exclusive media, we shall, after having tested the known species upon each, be able to state positively that any growth which appears upon any given medium is a member or members of a limited number of types belonging to that group (designated by media). Furthermore, we shall be able to plate out a soil, a milk, a water, etc., on a series of media and know, not only the number of bacteria present but also the number of the different groups present.

The schematic arrangement presented is tentative and is limited to water soluble compounds. Many of the groups suggested may have no energy value or the chemicals used may be too toxic or too expensive for practicable purposes. Other groups and compounds will no doubt be suggested by further study. Likewise, it is possible that subdivisions of the above groups may be made by using different sources of ammonia, nitrate or cyanide, etc., or by modifying other nutrient constituents or by changing the reaction. Up to the present time 16 of the above media have been tested. One using oxalate as a source of energy was negative throughout, the others have given promising results. It is hoped, therefore, that these tentative statements will stimulate needed investigation along this line, and criticism is heartily invited.

# ON THE SIGNIFICANCE OF THE VOGES-PROSKAUER REACTION[1]

MAX LEVINE

*From the Laboratories of the Engineering Experiment Station and the Department of Bacteriology of the Iowa State College, Ames, Iowa*

Theobald Smith (1895) first called attention to the ratio of the gases evolved in the decomposition of glucose by *B. coli* and its relatives. He pointed out that whereas *B. coli* produced twice as much hydrogen as carbon dioxide, equal volumes of these gases were formed by *B. aerogenes*.[2] In consequence of the inaccuracies in the determination of the gases in the Smith tube, the gas ratio has been generally discarded as a differential criterion. However, the comparatively recent work of Harden in England, and particularly that of Rogers and his associates in the Dairy Division of the United States Department of Agriculture, indicates that the gaseous and other decomposition products of glucose, if accurately determined, are of considerable importance in the differentiation of coli-like bacteria.

In a careful quantitative study of glucose fermentation, Harden and Walpole (1905), showed that *B. coli* evolved carbon dioxide and hydrogen in approximately equal volumes, and not, as had been observed by Smith, in the ratio of 1 to 2. On the other hand, the *B. aerogenes* formed twice as much carbon dioxide as hydrogen instead of the equal volumes observed with the Smith tube. They point out that the difference between the gas ratio obtained with the Smith tube and their accurately determined ratio is due to the loss of carbon dioxide in the former case owing to its solubility in the medium.

[1] Presented at the Seventeenth Annual Meeting of the Society of American Bacteriologists, Urbana, Illinois, December 28, 1915.

[2] *B. aerogenes* as employed in this paper, is synonymous with *B. lactis aerogenes*, i.e., those organisms of MacConkey's type IV (sucrose +, dulcitol −) which give a positive Voges-Proskauer reaction.

The real significance of the accurately determined gas ratio was not appreciated until 1914 when Rogers called attention to the striking correlation between this ratio and the source of the organisms. In three papers by Rogers, Clark and Davis, (1914) and Rogers, Clark and Evans (1914 and 1915), it is demonstrated quite conclusively that fecal strains of *B. coli* (at least those derived from bovine feces) break down glucose with the liberation of carbon dioxide and hydrogen in about equal volumes, while non-fecal (grain) strains form two or more times as much carbon dioxide as hydrogen. The sanitary significance of such a division is evident, but the accurately determined gas ratio is inapplicable to routine work.

Clark and Lubs (1915) note that the gas ratio is correlated with the $H^+$ ion concentration and that the difference in $H^+$ ion concentration between the low and high ratio groups is such that it may be easily recognized by methyl red. When grown in appropriate glucose media the low ratio, (fecal) group is acid and the high ratio (non-fecal) group alkaline to this indicator.

As no earlier investigators employed the methyl red reaction, the valuable work of these men cannot be compared directly or adequately with former investigations, unless some previously employed test is found which is well correlated with either the gas ratio or the methyl red test. The Voges-Proskauer reaction seems to serve this purpose.

## THE VOGES-PROSKAUER REACTION

The chemistry of the Voges-Proskauer reaction has been worked out in detail by Harden and his associates in England. West refers to one of Harden's articles in which it is pointed out that the reaction is due to the production of acetyl-methyl-carbinol and urges that this test be studied further, as it is of considerable importance in recognizing *B. aerogenes* and *B. cloacae*. Among other investigators who have employed this reaction in studies on *B. coli* may be mentioned, Durham, MacConkey, Rivas, Bergey and Deehan, Ferriera, Horta and Paredes, Copeland and Hoover, Clemesha, Archibald and more recently Kligler and Levine.

The significance of this reaction has not been fully appreciated by bacteriologists, nor has it been generally realized that the test is due to a definite end product of glucose fermentation. It will therefore not be amiss to review somewhat in detail the nature and chemistry of the Voges-Proskauer reaction.

The reaction takes its name from the fact that it was first observed by Voges and Proskauer in 1898, in their studies on the "Bacteria of Haemorrhagic Septicaemia." They describe the test as follows:

> On addition of caustic potash, we observed a new and interesting color reaction. If the tube be allowed to stand 24 hours and longer at room temperature, after the addition of the potash, a beautiful fluorescent color somewhat similar to that of a dilute alcoholic solution of eosin forms in the culture fluid particularly at the open end of the tube exposed to the air. We have investigated a few of the properties of this coloring substance, which is not produced by the action of the alkali on the sugar, and have found that it is fairly resistant to the action of the external air. After a time however, it becomes paler, and finally gives place to a dirty greenish brown.

It has been repeatedly observed in this laboratory, that, with some cultures, a distinct coloration which may be observed about five hours after addition of the potash fades or disappears entirely after twenty-four to forty-eight hours.

In a study of the end products of the fermentation of glucose by *B. coli*, Harden and Walpole (1905–06) observed that the products ordinarily enumerated, (lactic, acetic, succinic and formic acids, ethyl alcohol and carbon dioxide) do not account for all of the carbon in the sugar. Aside from these substances, a crude glycol was also obtained. This crude glycol consists for the most part of 2:3 butyleneglycol ($CH_3$-CHOH-CHOH-$CH_3$). On oxidation it yields acetyl-methyl-carbinol ($CH_3$, CHOH.CO.$CH_3$), a volatile reducing substance, which, when mixed with potassium hydroxide in the presence of peptone, imparts an eosine-like coloration to the mixture on standing. Butyleneglycol is oxidized to acetyl-methyl-carbinol by *B. aerogenes*, but not by *B. coli*. Harden (1905) ascribes the Voges-Proskauer reaction to the production of this carbinol.

Walpole (1910) found that in the presence of oxygen *B. aerogenes* gave a larger yield of acetyl-methyl-carbinol from glucose and that fructose was decomposed in a similar manner.

Neither acetyl-methyl-carbinol nor butyleneglycol, when mixed with potassium hydroxide give the eosin-like coloration. In the presence of peptone, however, the coloration develops on standing in the case of the carbinol, but not with the glycol. According to Harden (1905) the reaction is due to the further oxidation of the carbinol ($CH_3CO.CHOH.CH_3$) to diacetyl ($CH_3CO.CO.CH_3$) which reacts with some constituent of the peptone. In a later study Harden and Norris (1911) report that in the presence of strong potassium hydroxide solution diacetyl reacts with proteins to give a pink coloration together with a green fluorescence. With arginine, creatine, dicyanamide and guanidine acetic acid, the pink coloration is also obtained but the fluorescence is absent. The reaction depends on the presence of the group NH:C ($NH_2$) N:HR. The exact significance of R. has not been determined.

Among the organisms capable of forming acetyl-methyl-carbinol from carbohydrates may be mentioned, *B. aerogenes* Escherich, *B. cloacae* Jordan, *B. subtilis* Cohn, *B. vulgatus* Flügge. Peré obtained volatile substances which reduced Fehling's solution, by the aerobic fermentation of mannitol by *B. subtilis* and *B. vulgatus* and of glucose and glycerol by *Tyrothrix tenuis*.

## CORRELATION OF VOGES-PROSKAUER AND METHYL-RED REACTIONS

A study of 167 coli-like bacteria obtained from various sources including raw and septic sewage, stock cultures from the American Museum of Natural History, and feces of the horse, cow, sheep, hog and man, showed that only those which were alkaline to methyl red (in a medium made up of 0.5 per cent $K_2$ H $PO_4$, peptone and glucose) gave the Voges-Proskauer reaction. Of 13 cultures which gave these reactions, 9 were from sewage and 4 from the museum collection. It should be noted that coli-like organisms giving these reactions were not obtained, even in a single instance, from the fecal samples.

In order to test further, the correlation between the $H^+$ ion concentration, and acetyl-methyl-carbinol production, 10 organisms which Dr. Kligler had observed to be positive for the Voges-Proskauer reaction were obtained. Two of these failed to form gas from glucose and will not be considered further here. The remaining eight strains were alkaline to methyl red and gave a positive Voges-Proskauer test.

Thirteen organisms were also obtained from L. A. Rogers. Five were acid to methyl red and gave a negative Voges-Proskauer test. One failed to grow at 37°C. All of the others (7) were alkaline to methyl red and gave a positive reaction when tested for the formation of acetyl-methyl-carbinol in glucose-peptone solution.

The high correlation between the Voges-Proskauer reaction and the indicator test of Clark and Lubs makes it possible to compare the work of Rogers and his associates with that of previous investigators. Such a comparison shows a striking unanimity of opinion as to the significance of these reactions. Rogers regards the high gas ratio and alkalinity to methyl red as characteristic of *B. aerogenes*-like bacteria. Practically all who have employed the Voges-Proskauer reaction have pointed out that this test is characteristic of *B. aerogenes* and B. *cloacae*. Of a large number of coli-like strains examined by MacConkey in 1905, only three *B. aerogenes* Escherich *B. copsulatus* Pfeiffer and *B. cloacae* Jordan gave the Voges-Proskauer reaction. Durham in 1901 observed that this test was given only by those organisms which he regarded as belonging to the *B. aerogenes* group.

## THE DISTRIBUTION OF COLI-LIKE ORGANISMS WHICH GIVE A POSITIVE VOGES-PROSKAUER REACTION

Coli-like organisms which form acetyl-methyl-carbinol in glucose peptone solution are rarely found in feces. A reasonably accurate and reliable idea as to the distribution of such bacteria in nature may be obtained from a study of the distribution of *B. aerogenes* and *B. cloacae*.

MacConkey (1905) remarks on the scarcity of *B. aerogenes* in human feces. In the examination of 205 coli-like strains obtained from 22 samples of human stools, only 4 were *B. aerogenes* and of these 3 were isolated from a single sample. His observations on cow dung also indicated that this organism was extremely rare.

Ferriera, Horta and Paredes in an examination of 117 lactose fermenting strains from human feces obtained a positive Proskauer (presumably Voges-Proskauer) reaction in only eight instances. Among 81 coli strains obtained from 46 species of animals (8 birds and 38 mammals) they found only two which gave a positive "Proskauer" test.

The work of Clemesha is particularly significant because his conclusions are based on such large numbers of cultures. He examined 1207 organisms from human feces and 1029 from cow dung. In the latter *B. aerogenes* was found to be present in very small numbers and *B. cloacae* was sometimes common. In human stools, however, *B. aerogenes* and *B. cloacae* were very rare, nor was a sudden increase in the prevalence of these types ever observed. These findings are confirmed to a considerable extent by R. G. Archibald of the Wellcome Tropical Research Laboratories in an investigation of the water supply of Khartoum.

Of 117 cultures isolated in this laboratory from fecal sources (cow, horse, sheep, pig and man) not a single organism proved to be *B. aerogenes*, but of 39 organisms obtained from raw and septic sewage 9 (23 per cent) were of the *B. aerogenes* group (V.-P pos.). The relative abundance of these Voges-Proskauer positive organisms in sewage coupled with their extreme scarcity in human and other animal feces leads to the inference that they may perhaps represent soil forms.

Clemesha found that in India *B. aerogenes* is more prevalent in rivers and lakes during the wet reason than during the dry period. He explains this phenomenon on the basis of a supposed multiplication of the organisms in water, but observes that all his experiments indicate that such multiplication does not take place, at least in artificial mixtures. Nevertheless he maintains that in large bodies of water, such as rivers and lakes, there is un-

doubted multiplication of *B. aerogenes*. As to the prevalence of this organism he states, "In rivers, the period of time when rain is common is characterized by enormous increase in the number of *lactis-aerogenes* and yet we are perfectly certain that the organism is rare in feces."

These observations may be easily explained on the assumption that *B. aerogenes* is a soil form. In a study of coli-like microorganisms of the soil, now under way by B. R. Johnson and the author, preliminary tests have shown that a large proportion of cultures react positively for the Voges-Proskauer test, and are therefore of the *aerogenes-cloacae* group.

### ON THE FORMATION OF ACETYL-METHYL-CARBINOL FROM DIFFERENT CARBOHYDRATES AND ALCOHOLS

Acetyl-methyl-carbinol, like carbon dioxid and various acids is a product of carbohydrate metabolism. The fermentation of carbohydrates with acid and gas formation is generally accepted as a reliable basis for differentiation of *B. coli*. It is possible that a study of the production of acetyl-methyl-carbinol from various carbohydrates might also be of differential significance.

Harden and Norris obtained acetyl-methyl-carbinol by growing *B. aerogenes* or *B. cloacae* in peptone solutions containing glucose, fructose, mannose, galactose, arabinose, isodulcite, mannitol or adonitol, but this compound was not formed with glycerol ethyleneglycol, or acetaldehyde.

Ferriera, Horta and Paredes, studied the Proskauer reaction (presumably the Voges-Proskauer reaction) with glucose, galactose, maltose, lactose, sucrose, dulcitol, mannitol, and inulin. The reaction was positive eight times out of 117 tests with glucose, 7 times out of 48 tests with galactose, and twice in 48 tests with mannite. Practically all cultures gave traces with lactose and maltose while with dulcitol and inulin the reaction was invariably negative. These authors give an interesting table in which *B. coli*, *B. cloacae* and *B. aerogenes* are differentiated on the basis of the "Proskauer" reaction with different carbo-

hydrates and alcohols. It appears that *B. cloacæ* differs from *B. aerogenes* by the ability to give the reaction with mannite and galactose while the latter (*B. aerogenes*) may be distinguished from *B. coli* by its positive reaction in glucose and saccharose. The differentiation is suggestive and interesting but questionable, since the experimental evidence is inconclusive.

The term "Voges-Proskauer Reaction" is generally understood to mean the production of an eosin-like coloration when a glucose broth culture is made alkaline with potassium hydroxide. To employ the same term when some other carbohydrate is substituted for glucose may lead to confusion. It is therefore, suggested that the term "Voges-Proskauer Reaction" be restricted to designate the formation of acetyl-methyl-carbinol from glucose, but when referring to its production from other carbohydrates or alcohols, the term *Acetyl-methyl-carbinol Test* or merely *Carbinol Test* be employed. The nature of the substance being tested for is thus indicated just as is the case with the *Indol Test.*

The following experiment was carried out as a preliminary study on the production of acetyl-methyl-carbinol from various substances by coli-like bacteria. Forty-six organisms were selected. Twenty were strains which previous studies had shown did not produce the carbinol from glucose. They represent five sources, horse, sheep, cow, pig and man. From each source a culture representative of each of MacConkey's four groups was included. As far as possible no two cultures were from the same animal. The object of this selection was to obtain a group of Voges-Proskauer negative strains which would be likely to contain many different varieties.

The other 26 cultures were strains which previous tests had shown could form the carbinol from glucose. These included 9 strains isolated from sewage, and 17 strains obtained from Rogers and Kligler.

The organisms were inoculated into a medium consisting of 0.5 per cent di-potassium phosphate 0.5 per cent peptone and 0.5 per cent of the test substance. The Digestive Ferments Company peptone was employed for the test with glucose.

With all other test substances Wittes peptone was used. Incubation was at 37°C. for seventy-two hours.

Of the 26 supposedly Voges-Proskauer positive organisms, 4 failed to give the reaction in this experiment. Whether this phenomenon is due to the difference in peptone, variation in period of incubation, or loss of physiological function needs to be further investigated. With one of these organisms, it was observed, about eight months ago, that the test for acetyl-methyl-carbinol was negative until the seventh day of incubation.

In the table below are summarized the results. The cultures are arranged in three groups. One group comprises those strains which, repeated tests have shown, do not form acetyl-methyl-carbinol from glucose, even on long incubation (7 days). Another group contains 22 strains which do form this carbinol from glucose. The third group includes the 4 cultures whose Voges-Proskauer reaction is questionable.

*Correlation between the formation of acetyl-methyl-carbinol in glucose peptone solution and in peptone solution containing other carbohydrates and alcohols*

| TEST FOR ACETYL-METHYL-CARBINOL IN GLUCOSE PEPTONE SOLUTION (VOGES-PROSKAUER REACTION) | POSITIVE (22 STRAINS) | | NEGATIVE (20 STRAINS) | | QUESTIONABLE (4 STRAINS) | |
|---|---|---|---|---|---|---|
| | No. | Per cent | No. | Per cent | No. | Per cent |
| Positive reactions with: | | | | | | |
| Fructose | 22 | 100.0 | 0 | 00.0 | 0 | 00.0 |
| Galactose | 20 | 90.9 | 0 | 00.0 | 1 | 25.0 |
| Maltose | 21 | 95.5 | 17 | 85.0 | 2 | 50.0 |
| Lactose | 15 | 68.2 | 5 | 25.0 | 0 | 00.0 |
| Sucrose | 21 | 95.5 | 1 | 5.0 | 1 | 25.0 |
| Raffinose | 8 | 36.4 | 0 | 00.0 | 1 | 25.0 |
| Mannitol | 13 | 59.1 | 0 | 00.0 | 0 | 00.0 |
| Glycerol | 0 | 00.0 | 0 | 00.0 | 0 | 00.0 |
| Salicin | 5 | 22.0 | 0 | 00.0 | 0 | 00.0 |
| Dextrin | 0 | 00.0 | 0 | 00.0 | 0 | 00.0 |

Practically all strains gave a trace of acetyl-methyl-carbinol with maltose. A very striking fact indicated in the table is that coli-like organisms which do not form acetyl-methyl-carbinol from glucose are characterized by an inability to produce this compound from the other substances tested. In only

one instance (5 per cent) was the carbinol test positive with sucrose; in 5 cultures (25 per cent) traces were observed with lactose; but with fructose, galactose, raffinose, mannitol and salicin the reaction was invariably negative.

On the other hand, the carbinol test with the organisms which gave a positive reaction with glucose was almost always positive with levulose (100 per cent), galactose (90.9 per cent), and sucrose (95.5 per cent), usually positive with lactose (68.2 per cent) and mannitol (59.1 per cent), and occasionally positive with raffinose (36.4 per cent) and salicin (22 per cent).

These results show quite conclusively that the metabolism of certain carbohydrates by the fecal group of coli-like organism is fundamentally different from that of the non-fecal group.

The carbinol test was usually most intense and distinct in sucrose peptone solution.

## CONCLUSIONS

The Voges-Proskauer reaction and alkalinity to methyl red in 0.5 per cent di-potassium phosphate, glucose peptone solution are correlated. These reactions were not given by any of the 117 strains isolated from the feces of the horse, cow, sheep hog and man.

A review of the literature on the distribution of *B. aerogenes* and *B. cloacae* (Voges-Proskauer positive organisms) corroborates the contention of Rogers and his associates that coli-like organisms which give a high $CO_2/H_2$ gas ratio, and an alkaline reaction to methyl red in 0.5 per cent peptone di-potassium phosphate glucose solution, are rare in feces.

The natural habitat of coli-like bacteria which form acetyl-methyl-carbinol from glucose and other carbohydrates is probably the soil.

The production of acetyl-methyl-carbinol from different carbohydrates and alcohols might serve as a differential index.

Practically all strains gave a trace of the carbinol in maltose-peptone solution.

Of the organisms which did not give the Voges-Proskauer reaction only 5 per cent formed acetyl-methyl-carbinol from sucrose, and 25 per cent gave a trace from lactose. With all

the other substances tested fructose, galactose, raffinose, mannitol, glycerol, salicin, and dextrin the carbinol test was negative.

Of the cultures which were positive for the Voges-Proskauer reaction, 100 per cent formed the carbinol from fructose, 90.9 per cent from galactose, 68.2 per cent from lactose, 95.5 per cent from sucrose, 36.4 per cent from raffinose, 59.1 per cent from mannitol and 22 per cent from salicin.

The Voges-Proskauer reaction is of considerable sanitary significance. It differentiates between fecal and non-fecal coli-like organisms and may be an index of soil washings.

## BIBLIOGRAPHY


ARCHIBALD, R. G. 1907. Lactose fermenting bacilli in surface waters, feces, etc. Wellcome Tropical Research Laboratories 4th Report, 319.

*BERGEY, D. H. AND DEEHAN, S. J. 1908. The *Colon-Aerogenes* group of bacteria. Journ. of Med. Res., *19*. 175.

CLARK, WM. M. AND LUBS, H. A. 1915. The differentiation of bacteria of the *Colon-Aerogenes* family by the use of indicators. Journ. of Inf. Dis., *17*. 160.

CLARK, WM. M. 1915. The final hydrogen ion concentrations of cultures of *Bacillus coli*. Journ. of Biol. Chem., *22*. 87.

CLEMESHA, W. W. 1912. The bacteriology of surface waters in the tropics. Calcutta and London.

CLEMESHA, W. W. 1912. A criticism of Dr. A. C. Houston's report on the biological characters of *B. coli* isolated from (1) raw, (2) stored river water, (3) stored and filtered water. Journ. of Hygiene., *12*, 463.

COPELAND, W. R. AND HOOVER, C. P. 1911. The interpretation of tests for *B. coli communis* Journ. of Inf. Dis., *9*, 343.

DURHAM, F. E. 1901. Some theoretical considerations upon the agglutinins, together with further observations upon *Bacillus typhi abdominalis*, *Bacillus enteritidis*, *Bacillus coli communis*, *Bacillus lactis aerogenes*, and some other bacilli of allied character. Journ. of Exp. Med., *5*, 353.

FERREIRA, A., HORTA, A., AND PAREDES, C. 1908. Recherches sur le *B. coli communis* de l'intestin de l'homme. Archivos do Real Instituto Bacteriologico Camara Pestana, Tome ii, Fasc. ii, 153.

FERREIRA, A. HORTA, A., AND PARADES, C. 1908. Recherches sur le B. coli de l'intestin des Mammiferes et des Oiseaux. Archivos do Real Instituto Bacteriologico Camara Pestana, Tome ii, Fasc. ii, 203.

HARDEN, A. 1901. Action of *B. communis* on dextrose. Trans. of Chem. Soc. (1901), 604.

HARDEN, A. 1905. Action on glucose of lactose-fermenting bacteria of feces. Journ. of Hyg. *5*, 488.


* Original article not available.

HARDEN, A. 1905–06. On the Voges-Proskauer Reaction for certain bacteria. Proc. Roy. Soc., *77*, 424.

*HARDEN, A., AND NORRIS, D. 1911–12. The bacterial production of acetyl-methyl-carbinol and 2:3 butylene-glycol from various substances. Proc. Roy. Soc. (B) *84*, 492.

HARDEN, A., AND NORRIS, D. 1912. The bacterial production of acetyl-methyl-carbinol and 2: 3 butylene-glycol from various substances. Proc. Roy. Soc. (B) *85*, 73.

HARDEN, A., AND NORRIS, D. 1911. The diacetyl reaction for proteins. Jour. of Physiol., *47*, 332.

HARDEN, A., AND WALPOLE, S. G. 1905–06. Chemical action of *B. lactis aerogenes*, (Escherich) on glucose. Production of 2:3 Butylene-glycol and acetyl-methyl-carbinol. Proc. Roy. Soc. (B), *77*, 399.

KLIGLER, I. J. 1914. Studies on the classification of the colon group. Journ. Inf. Dis., *15*, 187.

LEMOIGNE, M. 1912. Fermentation du sucre par le Bacillus subtilis. Production du 2.3-butylene-gylcol. Compt. Rend. *155*, 792.

MACCONKEY, A. 1905. Lactose fermenting bacteria in faeces. Journ. of Hyg. *5*, 333.

MACCONKEY, A. 1909. Further observations on the differentiation of lactose-fermenting bacilli with special reference to those of intestinal origin. Journ. of Hyg., *9*, 86.

PERÉ 1896. Cited by Harden and Norris. 1912. Ann. Inst. Pasteur, *10*, 417.

RIVAS. 1908. Cited by West.

ROGERS, CLARK AND DAVIS. 1914. Journ. of Inf. Dis., *14*, 411.

ROGERS, CLARK AND EVANS. 1914. The characteristics of bacteria of the colon type found in bovine feces. Journ. of Inf. Dis., *15*, 100.

ROGERS, CLARK AND EVANS. 1915. The characteristics of bacteria of the colon type occurring on grains. Journ. of Inf. Dis., *17*, 137.

SMITH, T. 1893. The fermentation tube with special reference to Anaerobiosis and gas production among bacteria. The Wilder Quarter-century Book, 197.

*SMITH, T. 1895. Ueber den Nachweis des Bacillus coli communis im Wasser. Centr. für Bakt., *18*, 494.

*THOMPSON, J. 1911. The chemical action of *B. cloacae* (Jordan) on glucose and mannitol. Proc. Roy. Soc. (B), *84*, 500.

*THOMPSON, J. 1913. The chemical action of *B. cloacae* (Jordan) on citric and malic acids in the presence and absence of oxygen. Proc. Roy. Soc. (B), *86*, 1.

VOGES AND PROSKAUER. 1898. Beitrag zur Ernahrungs physiologie und zur differential diagnose der Bakterien der hamorrhagishen septicamie. Zeit. für Hyg., *28*, 20.

WALPOLE, S. G. 1910–11. The action of *B. lactis aerogenes* on glucose and mannitol. The effect of free oxygen on their production. The action of *B. lactis aerogenes* on Fructose. Proc. Roy. Soc. (B), *83*, 272.

WEST, F. D. 1909. Notes on the Voges-Proskauer reaction for *Bacillus coli communis*. Am. Journ. Pub. Hyg., *19*, 227.

# STUDIES ON SOIL PROTOZOA AND THEIR RELATION TO THE BACTERIAL FLORA. II[1]

JAMES M. SHERMAN

*From the Bacteriological Laboratories of the Wisconsin Agricultural Experiment Station, University of Wisconsin*

## VI. THE EFFECT OF VOLATILE ANTISEPTICS UPON SOIL PROTOZOA

### *Introduction*

It is claimed by Russell and Hutchinson and their co-workers that soils partially sterilized with volatile antiseptics are entirely freed from protozoa. Hutchinson (1913) further claims that the larger types of the soil protozoa are killed by the treatment of soil with caustic lime. On the other hand, the results reported by Gainey (1912) and by Grieg-Smith (1911) indicate that the application of such amounts of volatile antiseptics as are used in practice does not exterminate the protozoa. Even if it be acknowledged that some types of the soil protozoa are able to resist the process of partial sterilization by antiseptics, we must still consider the contention of Russell and Hutchinson that the harmful factor is inactivated for a considerable period, when not exterminated. Further, the possibility exists that the kinds of protozoa most detrimental to the bacterial flora are peculiarly susceptible to the antiseptics. Since the greater part of the protozoan fauna of the soil is inactive, the mere survival of certain types is not necessarily important, but the effect of volatile antiseptics upon the *active soil protozoa*, on the other hand, would appear significant.

### *Experiments*

Tests were made of partially sterilized soils to determine the number of protozoa and also the types. These tests were made

[1]Continued from the Journal of Bacteriology, vol. i, no. 1, p. 35.

with pots each containing one kilogram of soil. The number of protozoa was determined by the dilution method, while the types were determined by the inoculation of 25 grams of soil into sterile hay infusion.

The effect of volatile antiseptics upon the active protozoa was determined by the treatment of soils with carbon bisulphide and toluene and by determining the number of protozoa one day after treatment and again after two months. The results (Table XXVI) show that the active protozoa are not exterminated and again multiply to numbers equivalent to those found in normal soils. *Monas sp.*, *Dimorpha radiata* and *Flagellate A* were all observed on the 1/10,000 dilutions after two months.

TABLE XXVI

*Effect of toluene and $CS_2$ on the soil protozoa*

| POT NO. | TREATMENT | NUMBER OF PROTOZOA PER GRAM | |
|---|---|---|---|
| | | 1 day | 60 days |
| 1 | 2 per cent toluene | Less than 10 | 10,00 |
| 2 | 2 per cent toluene | Less than 10 | 10,000 |
| 3 | 2 per cent $CS_2$ | Less than 10 | 10,000 |
| 4 | 2 per cent $CS_2$ | Less than 10 | 10,000 |

In another experiment toluene, carbon bisulphide and chloroform were used, and samples were taken at the end of one month to determine the number and types of protozoa present. In this test it was found that the protozoan fauna had not been simplified, as far as could be noted by microscopic examination, there being present a very complex mixture of ciliates, flagellates and amoebae. At the end of a month the active protozoa were again present in just as large numbers as are found in untreated soils.

In the foregoing tests the antiseptics used were left in the soil. It was also thought desirable to treat some soils by the method followed by Russell and Hutchinson. These workers usually employed 1 per cent toluene and then after one day spread the soil out to allow the antiseptic to evaporate. Four pots of soil were treated after this manner and four other pots

TABLE XXVII

*(Effect of toluene, $CS_2$ and $CHCl_3$ upon the soil protozoa (one month after treatment)*

| POT NO. | TREATMENT | PROTOZOA PER GRAM | TYPES OF PROTOZOA |
|---|---|---|---|
| 1 | 2 per cent toluene | 10,000 | C. F. A. |
| 2 | 2 per cent toluene | 10,000 | C. F. A. |
| 3 | 2 per cent toluene | 10,000 | C. F. A. |
| 4 | 2 per cent $CS_2$ | 10,000 | C. F. A. |
| 5 | 2 per cent $CS_2$ | 10,000 | C. F. A. |
| 6 | 2 per cent $CS_2$ | 10,000 | C. F. A. |
| 7 | 2 per cent $CHCl_3$ | 10,000 | C. F. A. |
| 8 | 2 per cent $CHCl_3$ | 10,000 | C. F. A. |
| 9 | 2 per cent $CHCl_3$ | 10,000 | C. F. A. |

C = Ciliates; F = Flagellates; A = Amoebae.

were treated with 1 per cent toluene but not evaporated. As is shown in Table XXVIII the results after one month were similar to those obtained in the other experiments.

TABLE XXVIII

*Effect of toluene left in and evaporated upon the soil protozoa (one month after treatment)*

| POT NO. | TREATMENT | PROTOZOA PER GRAM | TYPES OF PROTOZOA |
|---|---|---|---|
| 1 | Left in | 10,000 | C. F. A. |
| 2 | Left in | 10,000 | C. F. A. |
| 3 | Left in | 10,000 | C. F. A. |
| 4 | Left in | 10,000 | C. F. A. |
| 5 | Evaporated | 10,000 | C. F. A. |
| 6 | Evaporated | 1,000 | C. F. A. |
| 7 | Evaporated | 10,000 | C. F. A. |
| 8 | Evaporated | 10,000 | C. F. A. |

Another test was made in which large amounts of volatile antiseptics were used in order to see if the protozoa could be entirely eliminated from soil. Soils were treated with 5 and 10 per cent of toluene and carbon bisulphide and the antiseptics left in the soil. Even with such large amounts of antiseptics the protozoa were not entirely eliminated, although the fauna was considerably simplified, especially with carbon bisulphide. As is shown in Table XXIX, ciliates, flagellates and amoebae

were found in every case except in the one treated with 10 per cent carbon bisulphide in which no amoebae were observed. In all of these soils *Monas sp.*, *Dimorpha radiata* and *Flagellates A* and *B* were present.

TABLE XXIX

*Effect of large amounts of toluene and carbon bisulphide upon the soil protozoa*

| TREATMENT | 5 PER CENT TOLUENE | | | 10 PER CENT TOLUENE | | | 5 PER CENT $CS_2$ | | | 10 PER CENT $CS_2$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Types of protozoa..... | C. | F. | A. | C. | F. | A. | C. | F. | A. | C. | F. |

Several attempts were made without success to demonstrate a stimulation of the protozoa, similar to that of the bacteria, subsequent to the application of volatile antiseptics to the soil. Moore (1912) in an address on the "Micro-organisms of the Soil" stated that results obtained in his laboratory indicated that the protozoa in soil not only withstood the action of antiseptics but that they might be increased by such treatment. Woodruff (1908) has shown that the multiplication of infusoria may be stimulated by small doses of alcohol. The dilution method for the determination of the number of protozoa is far too crude to measure small differences so the fact that it failed to demonstrate any increase in the number of protozoa following the application of volatile antiseptics to soil cannot be considered of much importance.

## *Discussion*

From the results herein reported it may be concluded that volatile antiseptics in the amounts used in practice do not free the soil from protozoa. The active soil protozoa not only survive, but multiply rapidly and again attain their normal numbers, usually within a month after treatment. It is difficult to explain the failure of Russell and Hutchinson to find protozoa in the soils which they treated. They noted the survival of certain flagellates which they do not, however, associate with the "detrimental factor." The failure of these workers to find ciliates and amoebae may be due to insufficient samples. The ciliates and amoebae are greatly reduced by the treatment of

soil with volatile antiseptics; these organisms, being inactive in most soils, do not increase subsequently and so it is obviously necessary to use a larger sample in order to demonstrate their presence.

That the presence of protozoa in the partially sterilized soils used in this work was not due to contamination was shown by holding ten pots of sterilized soil under identical conditions for one month and then taking samples for protozoa. Nine of these pots were found to be free from protozoa, while the tenth contained one small flagellate.

These results argue strongly against the protozoan theory as an explanation of the phenomena of partial sterilization, but it cannot be said they positively disprove it, since, as was pointed out before, the particular kinds that are detrimental, if such exist, may be very sensitive to volatile antiseptics.

## VII. EXPERIMENTS RELATING TO THE POSSIBLE EXISTENCE IN SOIL OF A HARMFUL BIOLOGICAL FACTOR WHICH IS DESTROYED BY THE ACTION OF VOLATILE ANTISEPTICS

### *Introduction*

The experiments made in his part of the work were planned in an effort to determine whether the beneficial action of volatile antiseptics upon the soil bacteria is due to the destruction of a detrimental factor which is antagonistic to them. This problem was attacked in much the same way as was the study of the soils containing protozoa and free of protozoa (Part IV). If normal soils contain a bacterial-limiting factor while partially sterilized soils do not, it would seem that that fact could be quite definitely established by a comparison of the numbers of bacteria found in these soils when subjected to various conditions. It should also be easy to demonstrate the presence of this harmful factor by the reinfection of the partially sterilized soils with a small amount of untreated soil.

*The effect of volatile antiseptics upon the subsequent development of bacteria and protozoa in soil*

It was thought that some light might be thrown upon the protozoan theory by making bacterial and protozoal counts on soils subsequent to treatment with volatile antiseptics. If this theory is correct we would expect to find the greatest number of bacteria in partially sterilized soil at a time when the

TABLE XXX

*Effect of volatile antiseptics upon the bacteria and protozoa in soil*

| POT | TREATMENT | BACTERIA PER GRAM | PROTOZOA PER GRAM |
|---|---|---|---|
| | Fifteen days after treatment | | |
| 1 | Control | 15,000,000 | 20,000 |
| 2 | Control | 14,500,000 | 20,000 |
| 3 | 2 per cent toluene | 14,000,000 | 100 |
| 4 | 2 per cent toluene | 15,000,000 | 1,000 |
| 5 | 2 per cent $CS_2$ | 13,000,000 | 100 |
| | Thirty days after treatment | | |
| 1 | Control | 20,800,000 | 20,000 |
| 2 | Control | 20,200,000 | 20,000 |
| 3 | 2 per cent toluene | 48,000,000 | 20,000 |
| 4 | 2 per cent toluene | 49,300,000 | 20,000 |
| 5 | 2 per cent $CS_2$ | 44,400,000 | 20,000 |
| | Forty-five days after treatment | | |
| 1 | Control | 17,000,000 | 20,000 |
| 2 | Control | 21,000,000 | 20,000 |
| 3 | 2 per cent toluene | 45,000,000 | 20,000 |
| 4 | 2 per cent toluene | 46,000,000 | 20,000 |
| 5 | 2 per cent $CS_2$ | 110,000,000 | 20,000 |

protozoa are depressed. This, however, does not appear to be the case as is shown by the following experiment. Determinations were made of the numbers of bacteria and protozoa in treated and untreated soils at intervals of fifteen days after treatment. The results of this test are given in Table XXX.

This table shows that the maximum number of bacteria is not found while the protozoa are depressed, but rather that

the development of the two classes of micro-organisms subsequent to treatment with volatile antiseptics runs parallel. This experiment was verified by another test in which normal and carbon bisulphide-treated soils were compared. In this test (Table XXXI) the number of bacteria in the treated soil rose above that of the control soil by the fifteenth day, but at this period the protozoa in the treated soil had also returned to their normal level. It is seen also that the number of bacteria continued to increase after the protozoa had again become as numerous as in untreated soil.

TABLE XXXI

*Effect of $CS_2$ upon the bacteria and protozoa in soil*

| POT | TREATMENT | FIFTEEN DAYS | | THIRTY DAYS | |
|---|---|---|---|---|---|
| | | Bacteria | Protozoa | Bacteria | Protozoa |
| 1 | Control..... | 23,000,000 | 10,000 | 60,000,000 | 20,000 |
| 2 | 2 per cent $CS_2$..... | 94,000,000 | 20,000 | 240,000,000 | 20,000 |

### *The reinoculation of partially sterilized soils*

In their work at the Rothamsted Station Russell and Hutchinson (1913) claim to have demonstrated that the soil contains a detrimental factor since the bacterial content of partially sterilized soil may be reduced by reinoculation with untreated soil. It is pointed out that when soil treated with a volatile antiseptic is reinoculated with 5 per cent of untreated soil the number of bacteria is reduced, while if only 0.5 per cent of normal soil is added no such reduction takes place. These observations are explained by the assumption that when only 0.5 per cent of untreated soil is added the harmful factor is not transmitted, but when 5 per cent is used for the inoculum the treated soil again becomes infected with the undesirable group of organisms. The soundness of this view may certainly be questioned, as it is difficult to understand why it should be necessary to use such a large amount of untreated soil in order to insure the presence of a factor which is supposed to exist in amount sufficient to suppress the bacteria. A review of the work of Russell

and Hutchinson reveals the fact that in some of the tests the treated soils which were reinoculated with 5 per cent of untreated soil did not show an appreciable depression in the number of bacteria, and they qualify their conclusion on this point with the statement that, "the harmful factor is not invariably transmitted to the same extent from the untreated to the partially sterilized soil and in a few cases indeed it is not transmitted at all."

In the experiments which were carried out in this laboratory the partially sterilized soils were reinoculated with 1 per cent of untreated soil; since at least 1 kgm. of soil was used in each pot the inoculum never consisted of less than 10 grams of normal soil. It could hardly be doubted that this amount of soil would be sufficient to transplant the group of organisms, if such exist, which act as a limiting factor upon the bacterial flora.

The work which has been done on the reinoculation of partially sterilized soils (Tables XXXII to XXXIV) fails to give any indication that a harmful factor is thus introduced. It would appear, on the other hand, that if reinfection of the treated

TABLE XXXII

*Effect of reinoculation of treated soil with untreated soil (treatment of 2 per cent toluene)*

| POT | TREATMENT | NUMBER OF BACTERIA PER GRAM | | |
|---|---|---|---|---|
| | | 30 days | 60 days | 90 days |
| 1 | Control | 56,000,000 | 80,000,000 | 69,000,000 |
| 2 | Control | 66,000,000 | 75,000,000 | 62,000,000 |
| 3 | Reinoculated | 57,000,000 | 82,000,000 | 79,000,000 |
| 4 | Reinoculated | 62,000,000 | 100,000,000 | 92,000,000 |

TABLE XXXIII

*Effect of reinoculation of treated soil with untreated soil (treatment 1 per cent toluene: evaporated)*

| POT | TREATMENT | NUMBER OF BACTERIA PER GRAM | |
|---|---|---|---|
| | | 15 days | 30 days |
| 1 | Control | 149,000,000 | 95,000,000 |
| 2 | Control | 127,000,000 | 81,000,000 |
| 3 | Reinoculated | 152,000,000 | 130,000,000 |
| 4 | Reinoculated | 178,000,000 | 92,000,000 |

TABLE XXXIV

*Effect of reinoculation of treated soil with untreated soil (treatment 2 per cent $CS_2$)*

| POT | CONTROL | AVERAGE | REINOCULATED | AVERAGE |
|---|---|---|---|---|
| 1 | 273,000,000 | 255,300,000 | 247,000,000 | 392,000,000 |
| 2 | 218,000,000 | | 317,000,000 | |
| 3 | 285,000,000 | | 422,000,000 | |

Incubation period after reinoculation: 2 months.

soil has any effect it is to increase the number of bacteria rather than to decrease it. However, the data on this point are doubtless within the boundaries of experimental error. It is difficult to reconcile these findings with the theory of Russell and Hutchinson.

### *The number of bacteria in partially sterilized and normal soils at different temperatures*

One of the strongest points in the evidence produced by Russell and Hutchinson to prove that the soil contains a harmful biological factor was the difference in the behavior of untreated and partially sterilized soils when incubated at different temperatures. Their results indicated that the maximum development of bacteria in the untreated soil was at low temperatures (5° to 12° C.), while in treated soil the greatest number was found at 20°C., and at 30°C. there was a marked increase over that found at 12°C.—the maximum in the case of the untreated soil. This phenomenon they claim shows that the bacteria under normal conditions are limited by the detrimental factor and that their maximum development takes place under conditions unfavorable for the harmful factor.

This point has been tested by the comparison of toluened and normal soils at 10°, 22°, and 37°C. The treated soil used had been treated with 2 per cent toluene three months previously. These soils were incubated for one month at their respective temperatures and then sampled and their bacterial counts determined. The results are given in Table XXXV.

TABLE XXXV

*The number of bacteria in treated and untreated soils at different temperatures*

| TREATMENT | NUMBER OF BACTERIA PER GRAM | | |
|---|---|---|---|
| | 10° C. | 22° C. | 37° C. |
| Untreated | 21,000,000 | 23,000,000 | 22,000,000 |
| 2 per cent toluene | 64,000,000 | 49,000,000 | 36,000,000 |

These data are not sufficient to base any conclusions upon but it can not be said they indicate very much, either in favor of the protozoan theory or against it. It will be seen that the greatest difference in the numbers of bacteria in the treated and untreated soils was at 10°C., a point not in favor of the protozoan theory. On the other hand, the least difference was found at 37°C., which point may support the theory of Russell and Hutchinson.

It was decided to carry out another experiment at 37°C. in order to throw more light on this point. Instead of using soils which had been previously treated, the soils were first placed at 37°C. and allowed to incubate at that temperature for one month. Half of them were then treated with 2 per cent carbon bisulphide. If the protozoan theory is correct the antiseptic should have very little effect at this high temperature. One month after treatment bacterial counts were made. The results obtained are given in Table XXXVI.

TABLE XXXVI

*Effect of $CS_2$ upon the number of bacteria in soil at* 37°C.

| POT | NUMBER OF BACTERIA PER GRAM | | | |
|---|---|---|---|---|
| | Untreated | Average | 2 per cent $CS_2$ | Average |
| 1 | 21,000,000 | 21,000,000 | 208,000,000 | 228,000,000 |
| 2 | 21,000,000 | | 248,000,000 | |

The results are very striking; a difference of over ten fold in the number of bacteria in the treated and untreated soils being found. This observation indicates strongly that the beneficial action of volatile antiseptics in soil is not to be explained by

its effect upon the protozoa. Soil extract and hay extract cultures made from untreated soil and incubated at 37°C. have failed entirely to reveal the presence of any of the active types of protozoa which have been mentioned as especially abundant in soil. In such cultures only a very few types of protozoa appear at all and these only slowly and in small numbers.

*The number of bacteria developing in sterilized soils reinoculated with untreated and with partially sterilized soils*

The preceding experiments appear to demonstrate quite conclusively that the beneficial effect of volatile antiseptics in soil is not due to the destruction of a biological factor, unless it be assumed that the treatment of soil so changes it that the harmful organisms are no longer able to develop in it, even though it is reinoculated with them. An experiment was planned in order to see if this explanation is a true one. Two pots of sterile soil were inoculated with 1 per cent of normal soil, while two other pots were inoculated with 1 per cent of a soil which had been treated with 2 per cent toluene. In case the antiseptic really destroys a harmful factor that fact should be indicated by a much greater number of bacteria in the soils inoculated with the treated soil. This result, however, was not obtained; on the contrary, the counts made at thirty and forty-five days after inoculation showed no practical difference between the numbers of bacteria in the two soils, as is shown in Table XXXVII.

TABLE XXXVII

*The number of bacteria developing in sterilized soils inoculated with normal and with toluened soils*

| POT | NUMBER OF BACTERIA PER GRAM | | | |
|---|---|---|---|---|
| | 35 days | | 45 days | |
| | Normal | Toluened | Normal | Toluened |
| 1 | 127,000,000 | 112,000,000 | 126,000,000 | 102,000,000 |
| 2 | 208,000,000 | 148,000,000 | 110,000,000 | 104,000,000 |
| Average..... | 190,000,000 | 130,000,000 | 118,000,000 | 103,000,000 |

*The effect of carbon bisulphide on the number of bacteria in sterilized soils reinoculated with normal soil and with protozoa-free soil*

Another experiment performed in order to detect the presence of the "harmful factor" was to inoculate soils sterilized by steam with normal soil and with the protozoa-free soil described in an earlier part of this paper. These soils were allowed to stand three weeks and were then treated with 1 per cent of carbon bisulphide. Bacterial counts were made before the soils were treated and then at fifteen and thirty days after treatment. According to the phagocytic theory, it would be expected that the number of bacteria in the soil inoculated with normal soil would subsequently be greatly increased while the soils inoculated with the protozoa-free soil should not be appreciably affected.

As in the previous experiments, the results of this test give no indication that there exists in soil a biological factor which is harmful to the bacterial flora. It will be seen upon examination of Table XXXVIII that the soils free of protozoa and those containing protozoa behaved in exactly the same way.

TABLE XXXVIII

*Effect of $CS_2$ upon sterilized soils inoculated with normal soil and with protozoa-free soil*

| POT | NUMBER OF BACTERIA IN MILLIONS PER GRAM | | | | | |
|---|---|---|---|---|---|---|
| | Before treatment | | 15 days | | 30 days | |
| | Without protozoa | With protozoa | Without protozoa | With protozoa | Without protozoa | With protozoa |
| 1 | 178 | 120 | 228 | 140 | 166 | 109 |
| 2 | 172 | 110 | 182 | 142 | 144 | 91 |
| Average..... | 175 | 115 | 205 | 141 | 155 | 100 |

The results of this test add further weight to the preceding experiments all of which point to the non-existence of a detrimental biological factor in soil. The fact that volatile antiseptics have no appreciable effect in soils which have been sterilized by steam and then reinoculated with normal soil would appear to indicate that the beneficial effects derived by the use of these

substances are due to some action of the antispetics on the soil itself rather than to a simplification of its micro-organic population.

## VIII. RÉSUMÉ

### *Discussion*

The results of the foregoing experiments appear to establish quite definitely that protozoa in the soils which have been studied do not have a detrimental effect upon the bacterial flora. It is difficult to see how the action of an important phagocytic agent could have escaped detection by the methods employed unless the factor is unable to increase in soils which have been previously sterilized with steam or partially sterilized with volatile antiseptics when again introduced into these soils with an inoculum of normal soil. This restricted power of growth would be very different from the properties of micro-organisms in general, either of animal or plant nature, and there is no evidence, as far as we are aware, that a group of organisms with such peculiar characteristics exists in the soil. As has been pointed out, the soil protozoa, at least those types which appear in liquid cultures, grow better in soil which has been previously subjected to steam sterilization just as do the bacteria. Aside from the evidence that soil does not contain a biological factor which is inimical to the bacterial flora, the facts that volatile antiseptics do not exterminate the soil protozoa, and that partial sterilization of soil under conditions unfavorable for the action of protozoa (e.g., at 37°C.) is followed by the characteristic rise in the number of bacteria, would appear to cast serious doubt upon the theory of Russell and Hutchinson as an explanation for the effect of volatile antiseptics upon the soil bacteria.

Cunningham (1914) has recently published the results of some work which he thinks proves that protozoa act as a limiting factor upon the bacterial flora in soil. The fact that his data on this point are derived from only two experiments, one of which gave negative results, would preclude his conclusions

from very serious consideration. A study of the methods he used indicates, however, that the difference found in the soils with and without protozoa might have been due to a difference in the complexity of the two flora, as was the case in the experiments reported in Part IV (see Table XI) of this paper. In fact, his manner of attack was very similar; sterilized soils were employed as a substratum, and inoculations were then made into these soils of cultures containing protozoa and free of protozoa. "One flask was inoculated with bacteria plus protozoa from a culture of protozoa from soil, the other received as nearly as possible an equal inoculation from the same culture of bacteria alone." It is not clear from this statement how he obtained the bacterial culture free from protozoa, but it is very certain that a protozoa-free culture could not be obtained which would contain as complex a bacterial flora as did the original culture from which it was derived. As was previously pointed out, a difference in the complexity of the bacterial flora in different soils may cause a great disparity in the counts obtained by the plate culture method. This fact was apparently not recognized by Cunningham as he concluded that "the reduction in bacterial numbers in the soils inoculated with protozoa is very marked and lies well outside the limits of experimental error." A review of the data in Part IV of this paper will show, on the contrary, that his results may fall well within the limits of experimental error.

It is believed that the conclusions drawn from the work herein reported will hold in general for the cultivated soils in this country, but it is not desired to make too broad an application of them. Many of the "sick" soils which have been studied at the Rothamsted Experimental Station are very different from the ordinary American soil. Martin and Lewin (1914) describe a sick cucumber bed which was made up of one part of light pasture soil, one part of heavy pasture soil and two parts of horse manure, and had an optimum moisture content of 62 per cent. The assumption that the biological conditions in such a soil are the same as in the average soils of the United States (which contain about 2 per cent organic matter and the optimum

moisture content of which is from 16 to 18 per cent) would be obviously unwarranted. That a difference in the micro-fauna does exist under various soil conditions is indicated by the fact that Martin and Lewin have found amoebae to be the predominating types of protozoa in the soils they have studied, which are very rich in organic matter, while the results reported here, as well as the data obtained by Cunningham on German soils, indicate that the flagellates occur in greater numbers than do the amoebae. It appears possible that in the rich soils and green-house beds, which have been studied extensively at the Rothamsted Station in connection with soil sickness, there might be a phagocytic agent which is not active in ordinary soils. This possibility, however, should not make us unmindful of the fact that no direct evidence has as yet been produced which indicates that such a factor exists in any cultivated soil. It should also be remembered that the beneficial effects of partial sterilization of soil—for the explanation of which the protozoan theory was advanced—have been observed in all localities in which the problem has been studied and in nearly all types of soil.

The question of the activities of the protozoa which lead an active existence in soil is a problem upon which much work could profitably be done. The active protozoa which occur in soils in large numbers certainly have functions there, some of which in fact may be very important. It is not desired to give the impression that because the protozoa which have been studied do not exert a limiting action on the bacteria in soil that it is thought that they do not ingest bacteria at all. Some in all probability do not, while others (e.g., *Monas*) it would appear undoubtedly do. Why active protozoa which feed upon bacteria should not cause a measurable decrease in the number of bacteria in soil is difficult to explain. It would seem that the excretory products of the protozoa which feed upon the soil bacteria would increase the amount of available energy for the rest of the bacteria so that a condition of metabiosis would be established which might offset the antagonistic action of the protozoa. This hypothesis does not appear unreasonable when it is remembered that the chief limiting factor upon the bacteria

in the soil is the food supply. In liquid cultures, on the other hand, the limiting factor is not the food supply but the accumulation of detrimental by-products; the number of bacteria soon reaches its maximum and then begins to decline gradually. It can readily be seen that if predatory protozoa are added to liquid cultures, in which the bacterial flora is in a comparatively inactive condition due to the presence of harmful by-products, a very striking reduction in bacterial numbers will be noted. Whatever the effect of protozoa on bacteria in solutions may be the results herein reported appear to indicate that under ordinary conditions they are not able to limit the bacterial flora when acting in soil.

## *Summary*

1. Determinations made by means of the dilution method indicate that the normal fertile soil has a protozoan content approximating 10,000 per gram.

2. In the soils studied the flagellates were the predominating type of protozoa and not the ciliates nor amoebae.

3. *Colpoda cucullus* appears to be the most widely distributed ciliate in soil and is occasionally found in numbers approximating 1,000 per gram.

4. Certain of the soil flagellates are active in soils of normal, and even subnormal, moisture contents.

5. Tests made with the ciliates *Colpoda cucullus, Balantiophorus elongatus* and *Oxytricha sp.* show that these organisms are not active under ordinary soil conditions.

6. *Colpoda cucullus* is probably active whenever the moisture content is much above normal, but not under ordinary conditions of moisture.

7. Active soil protozoa attain greater numbers when inoculated into previously sterilized soil than in normal soil.

8. Sterile soils when inoculated with normal soil and with an artificial soil culture which is free of protozoa show a difference in the total number of bacteria as determined by the plate culture method, due to a difference in the complexity of the two flora.

9. A great difference may exist in the number of bacteria as determined by the plate culture method, due to a difference in the complexity of the flora, between soils which are free of protozoa.

10. Experiments with soils containing protozoa and free of protozoa showed that the bacterial flora in the two soils behaved in exactly the same way when exposed to different conditions of temperature and moisture content.

11. The data obtained indicate that soil does not contain a biological factor which is harmful to bacteria.

12. Pure culture tests with the ciliates, *Colpoda cucullus* and *Balantiophorus elongatus*, showed that these organisms are very detrimental to bacteria in solutions. In soil, since the ciliates are inactive, they are unable to affect the bacterial flora.

13. Pure culture tests with four types of active soil flagellates showed that these organisms were not capable of limiting the number of bacteria when acting in soil. One of the cultures, however, had a very marked limiting action upon the bacteria when tested in soil extract.

14. Treatment of soil with the ordinary amounts of volatile antiseptics (1 to 2 per cent) does not appear to simplify the protozoan fauna. A complex mixture of ciliates, flagellates and amoebae is to be found in cultures made from soils partially sterilized with volatile antiseptics.

15. As much as 10 per cent of carbon bisulphide and toluene when added to soil fails to exterminate the protozoa entirely.

16. The active soil protozoa which are at first suppressed by treatment with volatile antiseptics soon begin to multiply so that they are again found in numbers equal to those of untreated soil within one month after treatment.

17. The maximum number of bacteria in partially sterilized soil is not found while the protozoa are suppressed but after they have again returned to their normal level. It appears that the development of these two classes of micro-organisms subsequent to treatment with volatile antiseptics runs parallel.

18. The reinoculation of partially sterilized soils with 1 per cent of normal soil fails to decrease the number of bacteria.

19. The treatment of soil with carbon bisulphide at 37°C. gives a very marked increase in the number of bacteria in the soils treated.

20. Sterilized soils which are reinoculated with normal soil and with partially sterilized soil show no essential difference in the numbers of bacteria which develop.

21. When volatile antiseptics are applied to sterilized soils reinoculated with and without protozoa no difference is to be noted between the behavior of the bacteria in the different soils.

22. No evidence has been obtained which indicates that the beneficial effect of partial sterilization is due to the elimination of a biological factor which is harmful to the bacteria.

Acknowledgment is due Professors E. G. Hastings, A. S. Pearse and E. B. Fred of the University of Wisconsin, from whom criticisms and suggestions have been obtained during the progress of this work.

## LITERATURE CITED

BEIJERINCK, M. W. 1896 Kulturversuche mit Amoeben auf festem Substrate. Centbl. Bakt. (etc.), Abt. 1, *19*, 257–267.

1897 Amoebenkultur auf festen Substraten. Centbl. Bakt. (etc.), Abt. Z, *21*, 101–102.

1901 Ueber oligonitrophile Mikroben. Centbl. Bakt. (etc.), Abt. 2, *7*, 561–582.

BERLINER, E. 1909 Flagellaten-Studien. Arch. für Protistenkunde, *15*, 297–325.

BOLLEY, H. L. 1910 Conservation of the purity of soils in cereal cropping. Science n. s., *32*, 529–541.

1913 The complexity of the micro-organic population of the soil. Science n. s., *38*, 48–50.

1913 Cereal cropping: sanitation, a new basis for crop rotation, manuring, tillage, and seed selection. Science n. s., *38*, 249–259.

BOTTOMLEY, W. B. 1911 Some effects of bacteriotoxins on soil organisms. Report British Assoc. for the Advancement of Science, 1911, 608.

BUDDIN, W. 1914 Partial sterilization of soil by volatile and non-volatile antiseptics. Jour. Agr. Sci., *6*, pt. 4, 417–451.

CALKINS, G. N. 1901 The protozoa. New York: MacMillan and Company.

CAMERON, F. 1910 Introduction to the study of the soil solution. Jour. Phys. Chem., *14*, 393–451.

CAUDA, A. and SANGIORO, G. 1914 Untersuchungen über die Mikrofauna der Boden aus Reisgegenden. Centbl. Bakt. (etc.), Abt. 2, *42*, 393–398.

CELLI, A. 1896 Die Kultur der Amoeben auf festem Sunstrate. Centbl. Bakt. (etc.), Abt. 1, *19*, 536–538.

CELLI, A., AND FIOCCA. 1894 Beitrage zur Amoebenforschung. Centbl. Bakt. (etc.), Abt. 1, *16*, 329–339.

CUNNINGHAM, A. 1914 Studies on soil protozoa II. Centbl. Bakt. (etc.), Abt. 2, *42*, 8–27.

CUNNINGHAM, A. AND LÖHNIS, F. 1914 Studies on soil protozoa I. Centbl. Bakt. (etc.), Abt. 2, *39*, 596–610.

EGOROV, M. A. 1908 The effect of carbon bisulphide on soils and plants. Zhur. Opuitn. Agron. *9*, 34–95. *Abs.* Expt. Sta. Rec., *20*, 518.

EMMERICH, R., LEININGEN, W. AND LOEW, O. 1912 Über Bodensäuberung. Centbl. Bakt. (etc.), Abt. 2, *31*, 466–477.

FRANCÉ, R. H. 1911 Studien über edaphische Organismen. Centbl. Bakt. (etc.), Abt. 2, *32*, 1–7.

FRANK, B. 1888 Ueber den Einfluss welchen das Sterilisiren des Erdbodens auf die Pflanzen-Entwickelung ausübt. Ber. d. Deutsch. Bot. Ges. (Generalversammlungs Heft) *6*, 87–98.

FRED, E. B. 1911 Über die Beschleunigung der Lebenstätigkeit höherer und niederer Pflanzen durch kleine Giftmengen. Centbl. Bakt. (etc.), Abt. 2, *31*, 185–245.

1915 Relation of carbon bisulphide to soil organisms and plant growth. Report of Director, 1914, Wis. Agr. Expt. Sta., Bul. 250, 18–19.

FROSCH, P. 1909 Beitrag zur Biologie saprophytischer Amoeben. Zeitschr. f. Krebsforschung. *8*, 183. *Abs.* Centbl. Bakt. (etc.), Abt. 1, *45*, 347–349.

GAINEY, P. L. 1912 The effect of toluol and $CS_2$ upon the microflora and fauna of the soil. Ann. Rpt. Mo. Bot. Garden, 1912, 147–169.

GOODEY, T. 1911 A contribution to our knowledge of the protozoa of the soil. Proc. Roy. Soc. (London), Ser. B, *84*, 165–180.

1914 A preliminary communication of three new proteomyxan rhizopods from soil. Arch. Protistenk., *35*, 80–102.

GRIEG-SMITH, R. 1911 The bacteriotoxins and the agricere of soils. Centbl. Bakt. (etc.), Abt. 2, *30*, 154–156.

1912 The inactivity of the soil protozoa. Proc. Linn. Soc. N. S. Wales, 1912, 655–672.

HILTNER, L. 1907 Über neuere Ergebnisse und Probleme auf dem Gebiete der landwirtschaftlichen Bakteriologie. Jahresber. d. Vereinig. f. Angew. Bot., *5*, 200–222.

HILTNER, L. AND STÖRMER, K. 1903 Studien über die Bakterienflora des Ackerbodens, mit besonderer Berucksicktigung ihres Verhaltens nach einer Behandlung mit Schwefelkohlenstoff und nach Brache. Arb. K. Gesundheitsamt. Biol. Abt., *3*, 445–528.

HUNTEMÜLLER, O. 1905 Vernichtung von Bakterien im Wasser durch Protozoen. Arch. für Hygiene, *54*, 89–100.

HUTCHINSON, H. B. 1913 The partial sterilization of soil by means of caustic lime. Jour. Agr. Sci. *5*, 320–330.

HUTCHINSON, H. B. AND MACLENNAN, K. 1914 The relative effect of lime as oxide and carbonate on certain soils. Jour. Agr. Sci. *6*, 302–322.

KILLER, J. 1913 Die Zählung der Protozoen im Boden. Centbl. Bakt. (etc.), Abt. 2, *37*, 521–524.

KOCH, A. 1899 Ueber die Ursachen der Rebenmudigkeit mit besonderer Berüchsichtigung der Schwefelkohlenstoffbehandlung. Arbeiten der Deutschen Landw. Gesellschaft, *40*, 7–44.

1911 Über die Wirkung von Äther und Schwefelkohlenstoff auf hohere und niedere Pflanzen. Centbl. Bakt. (etc.), Abt. 2, *31*, 175–185.

KORSHUN, S. W. 1907 Zur Frage der Verbreitung des Abdominal typhus durch Trinkwasser. Arch. für Hygiene, *61*, 336–347.

KRUGER, W. AND SCHNEIDEWIND, W. 1899 Ursache and Bedeutung der Salpeterzersetzung im Boden. Landw. Jahr., *28*, 217–252.

LIPMAN, J. G., BLAIR, A. W., OWEN, I. L. AND MCLEAN, H. C. 1910 Experiments relating to the possible influence of protozoa on ammonification in the soil. N. J. Agr. Expt. Sta. Bul. 248.

LODGE, C. A. AND SMITH, R. G. 1912 Influence of soil decoctions from sterilized and unsterilized soils upon bacterial growth. 24th Ann. Rpt. Mass. Agr. Expt. Sta., 126–134.

LOEW, O. 1911 Are protozoa concerned in soil sickness? Ann. Rpt. Porto Rico Agr. Expt. Sta., 1910, 15–17.

1913 Protozoan life in soils of Porto Rico. Ann. Rpt. Porto Rico Agr. Expt. Sta., 1912, 13–14.

LYON, T. L. AND BIZZELL, J. A. 1910 Effect of steam sterilization on the water soluble matter in soils. Cornell Agr. Expt. Sta. Bul. 275.

MARTIN, C. H. 1912 A note on the protozoa from sick soils, with some account of the life-cycle of a flagellate monad. Proc. Roy. Soc. (London), Ser. B, *85*, 393–400.

1913 The presence of protozoa in soils. Nature, *91*, 111.

MARTIN, C. H. AND LEWIN, K. R. 1914 Some notes on soil protozoa. Phil. Trans. Roy. Soc. London, Ser. B, *205*, 77–94.

MINCHIN, E. A. 1912 An introduction to the study of the protozoa. London: Edward Arnold.

MOORE, G. T. 1912 Micro-organisms of the soil. Science n. s., *36*, 609–616.

MÜLLER, P. E. 1887 Studien über die naturlichen Humusformen. Berlin: Julius Springer.

NÄGLER, K. 1909 Entwicklungsgeschichtliche Studien über Amöben. Arch. für Protistenk., *15*, 1–52.

NOBBE, F. AND RICHTER, L. 1904 Über die Behandlung des Bodens mit Äther, Schwefelkohlenstoff, Chloroform, Benzol und Wasserstoffsuperoxid und deren Wirkung auf das Wachstum der Pflanzen. Landw. Versuchs-stat. *60*, 433–448.

PECK, S. S. 1910 Some bio-chemical investigations of Hawaiian soils. Expt. Sta. of the Hawaiian Sugar Planter's Assoc. Bul. 34.

PFEFFER, T. AND FRANKE, E. 1896 Beitrag zur Frage der Verwertung elementaren Stickstoff durch den Senf. Landw. Versuchs-stat., *46*, 117–151.

PICKERING, S. U. 1910 Studies of the changes occurring in heated soils. Jour. Agr. Sci., *3*, 258–276.

1910 Plant growth in heated soils. Jour. Agr. Sci., *3*, 277–284.

RAHN, O. 1913 Methode zur Schätzung der Anzahl von Protozoën im Boden. Centbl. Bakt. (etc.), Abt. 2, *36*, 419–421.

RUSSELL, E. J. AND GOLDING, J. 1912 Investigations on "sickness" in soil. Jour. Agr. Sci., *5*, 21–47.

RUSSELL, E. J. AND HUTCHINSON, H. B. 1909 The effect of partial sterilization of soil on the production of plant food. Jour. Agr. Sci. *3*, 111–144.

1913 The effect of partial sterilization of soil on the production of plant food. Jour. Agr. Sci., *5*, 152–221.

RUSSELL, E. J. AND PETHERBRIDGE, F. R. 1912 Investigations on "sickness" in soil. Jour. Agr. Sci., *5*, 86–111.

SCHREINER, O. AND REED, H. S. 1907 Some factors influencing soil fertility. U. S. Dept. of Agr., Bur. of Soils, Bul. 40.

1907 Certain organic constituents of the soil in relation to soil fertility. U. S. Dept. of Agr., Bur. of Soils, Bul. 47.

SHERMAN, J. M. 1914 The number and growth of protozoa in soil. Centbl. Bakt. (etc.), Abt. 2, *41*, 625–630.

STONE, G. E. 1912 The present status of soil sterilization. 24th Ann. Rpt. Mass. Agr. Expt. Sta., 121–125.

STÖRMER, K. 1907 Über die Wirkung des Schwefelkohlenstoffs und ähnlicher Stoffe auf den Boden. Jahresber. d. Vereinig. f. Angew. Bot., *5*, 113–131.

TSUJITANI, J. 1908 Über die Reinkultur der Amoeben. Centbl. Bakt. (etc.), Abt. 1, *24*, 666–670.

WHITNEY, M. AND CAMERON, F. 1904 Investigations in soil fertility. U. S. Dept. of Agr., Bur. of Soils, Bul. 23.

WILSON, G. W. 1914 Studies of plant growth in heated soil. Biochem. Bul., *3*, 202–209.

WOLFF, M. 1909 Der Einfluss der Bewässerung auf die Fauna der Ackerkrume mit besonderer Berucksichtigung der Bodenprotozoen. Mitt. Kaiser Wilhelm Instit. für Landw. (Bromberg), 382–401.

1912 Über Bodenprotozoen. Centbl. Bakt. (etc.), Abt. 2, *33*, 314–320.

WOODRUFF, L. L. 1908 Effects of alcohol on the life cycle of infusoria. Biol. Bul., *15*, 85–104.

# ARE SPORE-FORMING BACTERIA OF ANY SIGNIFICANCE IN SOIL UNDER NORMAL CONDITIONS?[1]

H. JOEL CONN

*Agricultural Experiment Station, Geneva, New York*

Among the best known of the soil microörganisms are the spore-forming bacteria. They have been described as soil-bacteria ever since the first bacteriological investigations of soil were made; and a more thorough taxonomic study has been made of them than of any other bacteria except those which have sanitary significance. It is seldom, however, that they comprise more than 10 per cent of the total flora of soil. Hiltner and Störmer (1903)[2] recognized three groups of colonies upon gelatin plates made from soil: liquefiers, non-liquefiers and *Streptothrix* forms. The liquefiers averaged about 5 per cent of the total flora. The ordinary spore-forming bacteria in soil are all rapid liquefiers and must have been included in this 5 per cent mentioned by Hiltner and Störmer. Similar results have been obtained by various other investigators.

The spore-forming bacteria, *B. mycoides*, *B. cereus*, and *B. megatherium*,. are practically always present in soil and have always been considered characteristic and important soil organisms. These bacteria develop on gelatin or agar plates much more rapidly than those which comprise the other 90 to 95 per cent of the soil flora, and form large, striking colonies. They are among the largest of all bacteria and have an unusually interesting morphology, so it is not surprising that they have been studied most extensively of all the soil organisms growing

[1] Presented at Seventeenth Annual Meeting of the Society of American Bacteriologists, Urbana, Illinois, December 29, 1915.

[2] Hiltner, L. and Störmer, K. Studien über die Bakterienflora des Ackerbodens, mit besonderer Berücksichtigung ihres Verhaltens nach einer Behandlung mit Schwefelkohlenstoff und nach Brache. Kaiserliches Gesundheitsamt, Biol. Abt. Land- u. Forstw. 3; p. 445–545. 1903.

on ordinary media, in spite of the fact that they are not very abundant in soil. In nitrogenous culture media these bacteria grow rapidly and cause a vigorous amonification. For this reason they have been assumed to be the important ammonifiers of the soil.

This assumption was accepted as reasonable when I began to study the bacteria of soil. The first suspicion to the contrary came when it was noticed that the numbers of these spore-formers in the soil remained almost constant under all conditions, while the other bacteria varied in number according to the moisture content, aeration of the soil, or other conditions. The most natural explanation for this seemed to be that these bacteria lived over unfavorable conditions in the form of spores. It was soon realized, however, that this argument could not be carried to its logical conclusion without assuming that spore-formers were normally present in soil only as spores; in which case naturally their numbers would not vary.

A series of tests to investigate this matter has been made at the New York Experiment Station during the past year. The method used depended upon the fact that spores can resist higher temperatures than the vegetative forms. To determine the number of spores and vegetative rods present in any soil, one lot of diluted soil-infusion was plated in the ordinary manner, while a parallel lot of the diluted infusion was heated before plating for fifteen or twenty minutes at 75 to 85°. Then the colonies of the three spore-bearers, *B. mycoides*, *B. cereus* and *B. megatherium*, appearing on each set of plates, were counted. The colonies that developed from the heated infusion were assumed to arise from spores only; while in the case of the unheated infusion colonies might arise from vegetative rods as well.

The culture medium used in these tests was gelatin.[3] On this medium each of the three organisms investigated produced a fairly characteristic colony, so that it was ordinarily possible to distinguish them with little difficulty from non-spore-formers on the plates made from unheated infusion. Plates were in-

[3] Twelve per cent of Gold Label gelatine dissolved in tap-water and clarified with white of egg.

cubated at 18°C. for seven days. This length of incubation was necessary in order to allow the late colonies (particularly of *B. megatherium*) to appear. The chief disadvantage of such a long incubation was that *B. mycoides* and *B. cereus* often had time to liquefy the plate completely unless high dilutions were used. Dilutions of 1–20,000 and sometimes even 1–100,000 or 1–200,000 were necessary in order to avoid this trouble. At such dilutions the numbers of colonies of the spore-forming bacteria were so few that a long series of plates had to be made in order to obtain a reliable count; and even then no significance could be attached to variations in the count unless they were quite large.

In the first of these tests a temperature of 85° was used; but later it was learned that at temperatures only about 10° higher than this large numbers of the spores were killed and it was suspected that even 85° might destroy some of them. For this reason 80° was used instead for a while, and in the last tests 75° was used. To test the efficiency of this last temperature the bacteria developing on the plates after heating the infusion were studied, and it was found that nothing but spore-bearing bacteria had survived (leaving out of account an occasional colony of some non-spore-forming type that might easily be due to air contamination).

The greatest source of error in this method, which could not well be avoided, is the possibility that the bacteria investigated may occur in soil in clumps or chains instead of as isolated individuals. There is some evidence that clumps of bacterial spores can be broken up by the action of heat, which would tend to increase the count in the heated infusion provided clumps do occur in the soil. No increase of any appreciable amount has ever been observed, however; and indeed, so far as microscopical examinations of soil have been made, no evidence has been obtained of chains or clumps of organisms of this type. For this reason this possibility of error did not seem great enough to invalidate the conclusions.

A series of twenty-six tests was made. The results are given in the following table. The most striking fact to be observed

TABLE I

*Number of spore-forming bacteria in soil infusion, before and after heating. Determined by means of gelatin plates*

| DATE | TYPE OF SOIL,* AND STATE OF CULTIVATION | TEMPERATURE USED | NUMBER OF B. MYCOIDES, B. CEREUS AND B. MEGATHERIUM PER GRAM OF SOIL | | |
|---|---|---|---|---|---|
| | | | In unheated soil-infusion | In heated soil-infusion | Difference in favor of the unheated soil-infusion |
| 1915 | | | | | |
| Jan. 23 | Dunkirk silty clay loam. Potted. Fallow | 85° | 900,000 | 370,000 | +530,000 |
| Feb. 10 | Dunkirk silty clay loam. Cultivated | 85° | 850,000 | 600,000 | +250,000 |
| Feb. 19 | Dunkirk silty clay loam. Potted. Fallow | 85° | 900,000 | 430,000 | +470,000 |
| Feb. 25 | Dunkirk silty clay loam. Sod | 85° | 700,000 | 875,000 | −175,000 |
| Mar. 10 | Dunkirk silty clay loam. Sod | 85° | 425,000 | 40,000 | +385,000 |
| Mar. 11 | Dunkirk silty clay loam. Sod. Sub-soil | 85° | 625,000 | 550,000 | + 75,000 |
| Mar. 13 | Volusia silt loam. Cultivated | 85° | 550,000 | 275,000 | +275,000 |
| Sept. 16 | Dunkirk silty clay loam. Sod | 85° | 400,000 | 425,000 | − 25,000 |
| Sept. 21 | Dunkirk fine sand. Sod | 80° | 725,000 | 680,000 | + 45,000 |
| Sept. 21 | Dunkirk fine sand. Cultivated | 80° | 250,000 | 625,000 | −375,000 |
| Sept. 24 | Ontario fine sandy loam. Sod | 80° | 1,100,000 | 900,000 | +200,000 |
| Sept. 24 | Ontario fine sandy loam. Cultivated | 80° | 975,000 | 1,100,000 | −125,000 |
| Oct. 19 | Dunkirk silty clay loam. Freshly potted | 80° | 525,000 | 400,000 | +125,000 |
| Oct. 20 | Honeoye stony loam. Sod | 75° | 800,000 | 1,150,000 | −350,000 |
| Oct. 20 | Honeoye stony loam. Cultivated | 75° | 750,000 | 1,200,000 | −450,000 |
| Oct. 22 | Dunkirk gravelly loam. Sod | 75° | 425,000 | 550,000 | −125,000 |
| Oct. 22 | Dunkirk gravelly loam. Cultivated | 75° | 1,500,000 | 1,450,000 | + 50,000 |
| Nov. 9 | Dunkirk silty clay loam. Potted | 75° | 700,000 | 1,050,000 | −350,000 |
| Dec. 7 | Dunkirk silty clay loam. Potted | 75° | 800,000 | 650,000 | +150,000 |

| | | | | | |
|---|---|---|---|---|---|
| Dec. 7 | Dunkirk silty clay loam. Dried. Potted | 75° | 775,000 | 650,000 | +125,000 |
| Dec. 7 | Dunkirk silty clay loam. Manured. Potted | 75° | 1,350,000 | 1,050,000 | +300,000 |
| Dec. 7 | Dunkirk silty clay loam. Manured and dried. Potted | 75° | 1,050,000 | 1,000,000 | + 50,000 |
| Dec. 25 | Dunkirk silty clay loam. Potted | 75° | 925,000 | 525,000 | +400,000 |
| Dec. 25 | Dunkirk silty clay loam. Dried. Potted | 75° | 550,000 | 200,000 | +300,000 |
| Dec. 25 | Dunkirk silty clay loam. Manured. Potted | 75° | 1,600,000 | 1,450,000 | +150,000 |
| Dec. 25 | Dunkirk silty clay loam. Manured and dried. Potted | 75° | 400,000 | 375,000 | + 25,000 |
| | Average of first eight tests | 85° | 670,000 | 445,000 | +230,000 |
| | Average of ninth to thirteenth test, inclusive | 80° | 715,000 | 740,000 | − 26,000 |
| | Average of last thirteen tests | 75° | 894,000 | 869,000 | + 21,000 |
| | Average of last eighteen tests | 80°–75° | 844,000 | 833,000 | + 5,300 |
| | Average of all twenty-six tests | | 788,000 | 712,000 | + 76,000 |

* The soil nomenclature of the Bureau of Soils is used in this table. The soils mentioned are described in the Soil Survey of Ontario County, New York, published by this Bureau.

at first glance is the regularity of the numbers of these organisms in the unheated infusion. The highest count is 1,500,000 and the lowest 400,000. Compared with bacterial counts in general, these show remarkable regularity, especially when it is considered that the soils varied from poor sand to richly manured loam and that the counts were made on plates of such high dilution that comparatively few colonies were obtained on each plate. The counts obtained from the heated infusion are not quite as regular; but if the first eight tests are excluded—in which the use of 85° may have killed a few spores—there is scarcely any more variation than in the case of the unheated infusion.

Because of this regularity in the counts it is possible to obtain general averages that can be fairly compared with each other. The average count from the unheated infusion is 788,000, from the heated infusion 712,000. This slight difference indicates that there are very few if any of the organisms present in soil in a form that can be killed by the temperatures used. Studying the figures more closely it will be noticed that the greatest differences between the two counts occurred in the first eight tests, in which 85° was used. The average count in these first eight tests, unheated, was 670,000, while the average count, heated, was 445,000. In the last eighteen tests, however, both counts averaged nearly the name, 844,000 and 833,000, respectively.

A more careful analysis of the data yields similar results. The last column of the table shows the difference between the two counts with a plus sign before it if the count obtained from the unheated infusion was the higher, with a minus sign if that from the heated infusion was the higher. It will be seen that there are eighteen cases in which the plus sign is used, and in these cases the greatest difference was 530,000, or if the tests are excluded in which 85° was used, it is 400,000. On the other hand in the eight tests in which a greater count was obtained from the heated infusion there is one difference as large as 400,000. The average difference between the two counts is 76,000 in favor of the unheated infusion; while if the

first eight tests are excluded it is only 5,300, an almost negligible figure.

It might be concluded from these data that some vegetative forms do exist in normal soil and for this reaon a higher count was obtained eighteen times from the unheated infusion; while in the other eight cases a higher count was obtained from the heated infusion because clumps were broken up by the heat. It is improbable, however, that these two factors should ordinarily so nearly neutralize each other; nor is it possible, if this explanation is used, to account for the greater average difference in the first eight tests than in those in which lower temperatures were used. It seems more reasonable to explain most of the differences in either direction as lying within the experimental error—a perfectly plausible assumption in view of the high dilutions used;— or if this is not enough to explain all the cases in which the higher count was obtained from the unheated infusion, to assume that an occasional less resistant spore was killed by the temperatures employed. The evidence all seems to indicate that the three organisms investigated do not occur in soil under normal conditions as active vegetative forms, but as spores. It is true that there are other spore-forming bacteria in soil besides these three types, in regard to which definite data could not be obtained because their colonies are not characteristic enough to be recognized with certainty; but none of them are as constantly present as the three types studied, and what evidence is at hand suggests that the same facts are true in regard to them as in regard to *B. mycoides*, *B. cereus* and *B. megatherium*.

It stands to reason, however, that these bacteria, so universally present in soil, must grow and multiply under some natural conditions. It is known that they ordinarily thrive in the presence of organic matter; so it seemed not improbable that they would multiply if manure were added to soil. A single experiment has been undertaken to test out this point, but with negative results. In a pot of soil, mixed with a heavy application of fresh horse manure, kept under observation for two months, there was at first a very great increase in the number of non-

spore-bearers, but no appreciable multiplication of spore-formers; nor was there any decrease large enough to be detected in the number of actual spores. Meanwhile the odor of the soil was enough to show that ammonification was vigorous. It is perfectly possible that a repetition of this test might yield different results; but evidently this experiment did not furnish the right conditions for the growth of the spore-forming bacteria. Also it is plain that ammonification can take place without them.

These results leave our knowledge as to the significance of spore-forming soil bacteria in a rather unsettled state. It has been quite generally taken for granted in the past that they are active in soil and of great importance. Perhaps their striking appearance in plate culture has led to the assumption that they could grow equally vigorously in soil. Yet they comprise but a small part of the soil flora, and even at that they do not seem to be present in vegetative form under normal conditions. Spores are generally regarded as inert.

Never the less these spore-forming bacteria of the soil do not decrease in numbers, and spores cannot live forever. Their occurrence in soil cannot be due to accidental contamination, or their numbers would not be so constant. If it is true, as these results indicate, that they are of practically no importance under normal field conditions, it becomes a matter of much interest to learn under what conditions they can become active and multiply.

## SUMMARY

1. The number of spore-forming bacteria in soil is relatively constant and is about the same in all the soils studied. Three of the spore-forming bacteria aways present in soil—*B. mycoides*, *B. cereus*, and *B. megatherium*—were selected for the purpose of comparison, because their colonies on gelatin plates are quite readily distinguishable. The total number of these three organisms, as determined by means of gelatin plates, proved to be between 400,000 and 1,500,000 per gram in the soils studied. They always comprised less than 10 per cent and usually less than 5 per cent of all the colonies developing on gelatin.

2. When soil-infusion was heated before plating at a temperature (75–85°C.) high enough to kill the vegetative forms of bacteria, nearly if not quite as many colonies of these spore-forming bacteria developed as when it was plated unheated. In about one-third of the cases, indeed, their numbers were actually slightly higher on the plates made after heating; although all such differences undoubtedly lay within the limits of the experimental error. This suggests that these bacteria occur in normal soil as spores rather than in a vegetative state.

3. No increase in the total number of these organisms nor decrease in the number of their spores could be detected in a pot of soil to which fresh manure had been added.

4. These results throw considerable doubt on the common assumption that these organisms are important ammonifiers in the soil. They raise the question as to what possible soil conditions favor their growth and multiplication.

# A POSSIBLE FUNCTION OF ACTINOMYCETES IN SOIL[1]

H. JOEL CONN

*Agricultural Experiment Station, Geneva, New York*

It is not generally agreed whether *Actinomycetes* are to be classed with bacteria or with molds. They are thought to belong with the *Hyphomycetes* by some mycologists; but those that occur in the soil have generally been considered in connection with the bacterial flora rather than with the soil fungi. The reason why they have been studied by soil bacteriologists may be partly because *Actinomycetes* can be handled by much the same methods as the lower bacteria; and partly because both of these groups seem to be much more numerous than molds proper in normal soil.

The abundance of *Actinomycetes* in soil has been recognized for some time. In 1903 Hiltner and Störmer (1903) showed that of the colonies developing on gelatin plates from normal soil, 5 per cent were ordinarily liquefiers, 70 per cent non-liquefiers, and 20 per cent *Streptothrix* forms (a name often, although incorrectly, applied to this group). Probably everyone who has plated soil in gelatin, provided he has incubated his plates long enough for the slow-growing organisms to appear, will recognize these figures as typical of ordinary soil.

Perhaps the most interesting recent work on soil *Actinomycetes* is that of Krainsky (1914). It contains a valuable classification of these organisms and shows that the reason why few species have been distinctly recognized in the past is because the *Actinomycetes* require special media in order to bring out their specific characteristics. His further contention, however, that these special media are necessary in order to show the abundance of

[1] Presented at Seventeenth Annual Meeting of the Society of American Bacteriologists, Urbana, Illinois, December 29, 1915.

*Actinomycetes* in soil is not correct. With his special media he claims to have found as many as 20,000 per gram of soil; but he overlooks the fact that Hiltner and Störmer (1903) found as many as 2.5 millions per gram. Moreover, in the work that forms the basis of the present paper, 2 or 3 million per gram has proved to be a very common figure; while on certain occasions the number has reached 12 to 14 millions. Occasionally over half the colonies developing on gelatin have been *Actinomycetes* —this in spite of the fact that Krainsky claims their growth to be suppressed by ordinary media.

The great abundance of *Actinomycetes* in soil has led to many speculations as to their significance. It has often been stated that they are active agents in the decomposition of organic matter; but their part in this process has not been definitely studied. Beijerinck (1900) showed that one type was often present in the corky layer of various roots. He called this type *Streptothrix chromogena* after Gasperini (1894) (who, however, had called it *Actinomyces chromogenus*). This type is one of the most numerous in soil; yet in the light of recent work it must be regarded as a group rather than a species. To this group belongs the causal organism of potato scab. Lutman and Cunningham (1914), indeed, have recently attempted to show that the cause of this disease must be renamed *Actinomyces chromogenus* because it agrees in every particular with Gasperini's description of that organism. This is plainly impossible; for Krainsky (1914) has shown that at least four separate species agree with the descriptions that have been given to *A. chromogenus*.

This fact brought out by Krainsky is very evident to anyone who uses his methods for studying the group. In fact it has proved possible, by the use of other special media[2] besides those described by him, to recognize many more types than those listed in his article. Work is now in progress along this line.

[2] The medium which has given the best results of any yet investigated contains: 1000 cc. water, 15 g. agar, 10 g. glycerin, 1 g. sodium asparaginate, 1 g. glucose, 1.5 g. $NH_4H_2PO_4$, 0.2 g., $MgSO_4$, 0.1 g. $CaCl_2$, 0.1 g. KCl, trace $FeCl_3$. Further media are now being tested out that may prove even more satisfactory.

This complexity in the group and the confusion in nomenclature, however, must not hide the fact that an *Actinomyces* causes potato scab, nor that Beijerinck, approaching the subject from an entirely different angle, has shown them to be associated with the roots of other plants. It is also to be remembered that *Actinomycetes* are thought to be concerned in the decomposition of organic matter. Some recent observations at the New York Experiment Station bear on this point.

In the course of a qualitative study of the bacteria in certain New York State soils, it was early recognized that there was a great similarity between different soils in the relative numbers of *Actinomycetes* and lower bacteria present, provided the soils were in the same state of cultivation. Later it also became evident that the *Actinomycetes* were practically always present in greater abundance in old sod soil than in soil recently cultivated. This difference is shown in Table I, in which the numbers of *Actinomycetes* found in 20 samples of various sod soils are compared with the numbers occurring in an equal number of samples of cultivated soil. Although it is possible to pick out numerous cases in which the number occurring in some one of the cultivated samples is greater than in some of the sod samples, nevertheless the average number in sod soil is twice that in the cultivated soil. The table also shows that the *Actinomycetes* averaged 39.4 per cent of the total flora of sod soil, but only 21.3 per cent of the flora of cultivated soil. There is only one instance (October 22, 1913) in which the percentage of these organisms in sod soil is as low as their average percentage in cultivated soil, and only one (January 4, 1911) in which their percentage in cultivated soil is as high as their average percentage in sod soil.

These figures furnish a strong indication that *Actinomycetes* are more numerous in sod than in cultivated soil; but even before all the data given in Table I were collected the importance of making a more satisfactory comparison was realized. To do this, a study was made of a considerable variety of soil types,[3]

[3] The soil nomenclature of the Bureau of Soils has been used in this work. The soils mentioned are described in the Soil Surveys of Ontario and Tompkins Counties, New York, published by this Bureau.

TABLE I

*Number of Actinomycetes in miscellaneous samples of sod and cultivated soils. Determined by means of gelatin plates*

| SOD SOIL | | | | CULTIVATED SOIL | | | |
|---|---|---|---|---|---|---|---|
| Date | Soil type | Number per gram | Per cent of total flora | Date | Soil type | Number per gram | Per cent of total flora |
| Nov. 8, 1911 | Dunkirk fine sand..... | 1,000,000 | 28.0 | Feb. 7, 1910 | Dunkirk clay loam..... | 4,000,000 | 18.0 |
| Apr. 29, 1913 | Dunkirk fine sand..... | 3,800,000 | 38.0 | May 28, 1910 | Dunkirk clay loam..... | 2,000,000 | 29.0 |
| June 4, 1913 | Dunkirk sandy loam..... | 2,800,000 | 36.5 | Aug. 20, 1910 | Dunkirk clay loam..... | 2,000,000 | 23.0 |
| Oct. 27, 1913 | Muck..... | 33,000,000 | 28.0 | Jan. 4, 1911 | Dunkirk clay loam..... | 6,000,000 | 40.0 |
| Apr. 24, 1913 | Ontario fine sandy loam... | 8,000,000 | 36.5 | Feb. 8, 1911 | Dunkirk clay loam..... | 4,800,000 | 35.0 |
| May 7, 1913 | Loam. Type not known.. | 3,500,000 | 35.0 | Nov. 24, 1911 | Dunkirk silty clay loam.. | 6,300,000 | 21.5 |
| May 12, 1913 | Honeoye stony loam..... | 8,000,000 | 43.0 | Jan. 13, 1912 | Dunkirk silty clay loam.. | 6,000,000 | 17.5 |
| May 14, 1913 | Honeoye stony loam..... | 8,500,000 | 30.0 | Jan. 23, 1912 | Dunkirk silty clay loam.. | 2,800,000 | 25.0 |
| May 26, 1913 | Dunkirk fine sandy loam.. | 7,000,000 | 56.5 | Apr. 24, 1912 | Dunkirk silty clay loam.. | 3,800,000 | 16.5 |
| June 29, 1913 | Ontario loam..... | 7,500,000 | 34.5 | Apr. 24, 1912 | Volusia silt loam..... | 2,200,000 | 11.5 |
| July 11, 1913 | Heavy loam. Type not known..... | 12,800,000 | 38.5 | June 5, 1912 | Dunkirk silty clay loam.. | 3,200,000 | 16.0 |
| Sept. 15, 1913 | Dunkirk loam..... | 4,200,000 | 54.0 | June 5, 1912 | Volusia silt loam..... | 2,600,000 | 15.5 |
| Sept. 29, 1913 | Genesee silty clay loam... | 9,000,000 | 75.0 | June 20, 1912 | Volusia silt loam..... | 3,200,000 | 23.5 |
| Oct. 22, 1913 | Genesee loam..... | 9,500,000 | 21.5 | Sept. 7, 1912 | Volusia silt loam..... | 4,000,000 | 26.6 |
| Nov. 12, 1914 | Dunkirk silty clay loam... | 6,000,000 | 42.8 | Oct. 11, 1912 | Volusia silt loam..... | 1,200,000 | 11.0 |
| Nov. 25, 1914 | Dunkirk silty clay loam... | 8,300,000 | 37.8 | Sept. 28, 1912 | Dunkirk silty clay loam.. | 8,200,000 | 21.0 |
| Nov. 27, 1914 | Dunkirk silty clay loam... | 4,500,000 | 45.0 | Oct. 25, 1912 | Dunkirk silty clay loam.. | 3,300,000 | 17.5 |
| Feb. 25, 1915 | Dunkirk silty clay loam... | 10,500,000 | 23.8 | Dec. 3, 1912 | Dunkirk silty clay loam.. | 5,300,000 | 15 2 |
| Mar. 10, 1915 | Dunkirk silty clay loam... | 8,700,000 | 43.7 | July 0, 1913 | Dunkirk silty clay loam.. | 2,600,000 | 13.6 |
| Mar. 11, 1915 | Dunkirk silty clay loam... | 8,000,000 | 39.0 | Aug. 5, 1914 | Volusia silt loam..... | 3,000,000 | 30.0 |
| Average..... | | *7,750,000 | 39.4 | Average..... | | 3,800,000 | 21.3 |

* The first four counts in this column are omitted from the average. The total numbers of bacteria per gram are so low in sand and so high in muck that they cannot fairly be included with the rest.

and in order to avoid as many as possible of the other factors that might be involved in a comparison of miscellaneous soils two samples were always collected on the same date, from spots in the same soil not more than a few yards apart, one in old sod, the other in a cultivated field. In this series of tests thirty-eight pairs of samples were taken. Also a second shorter series of tests was made to compare the *Actinomyces* flora of three neighboring spots in a single soil type (Dunkirk silty clay loam), one spot in fallow soil, one in old sod and the third in a field which had been in grass for two or three years only.

All of the counts in these tests were made by means of gelatin plates, because in the earlier work gelatin had been found the best of the various media used for distinguishing *Actinomyces* colonies from those of the lower bacteria. The gelatin used sometimes contained soil-extract and sometimes tap-water alone.[4] Plates were always incubated for seven days at 18°C. before counting.

The results of the first series of tests are given in Table II. It will be seen that the average number of *Actinomycetes* in sod soil is nearly twice as high as the average number in cultivated soil and that they averaged 37.5 per cent of the total flora in sod soil but only 20.5 per cent of the flora of cultivated soil. These general averages are much like those given in Table I, but they tell only a part of the story, as it is possible for individual exceptions to obscure the differences in the average. In order to show the differences more plainly, the individual ratios were determined and averaged. In the sixth column of Table II is given the ratio of the actual number of *Actinomycetes* in the sod soil to the number in the corresponding samples of cultivated soil; in the last column of the table is given the ratio of the percentage of *Actinomycetes* in sod soil to the percentage in the corresponding samples of cultivated soil. A study of these ratios brings out some information not shown by the general averages.

[4] For the composition of these media see: Conn, H. J. Culture Media for Use in the Plate Method of Counting Soil Bacteria. N. Y. Agric. Exper. ta. Tech. Bul. 38, 1914.

TABLE II

*Number of Actinomycetes in neighboring sod and cultivated spots of various soil types. Determined by means of gelatin plates*

| DATE | SOIL TYPE | ACTUAL NUMBER PER GRAM | | | | PER CENT OF TOTAL FLORA | | |
|---|---|---|---|---|---|---|---|---|
| | | Sod | Cultivated | Difference | Ratio | Sod | Cultivated | Ratio |
| 1913 | | | | | | | | |
| Nov. 18 | Dunkirk fine sand | 3,600,000 | 3,000,000 | 600,000 | 1.2 : 1 | 45.0 | 33.0 | 1.4:1 |
| Nov. 20 | Ontario fine sandy loam | 10,000,000 | 4,000,000 | 6,000,000 | 2.5 : 1 | 37.0 | 19.0 | 1.9:1 |
| Dec. 1 | Dunkirk fine sand | 2,700,000 | 1,600,000 | 1,100,000 | 1.7 1 | 29.8 | 19.5 | 1.5:1 |
| Dec. 5 | Ontario fine sandy loam | 6,300,000 | 5,300,000 | 1,000,000 | 1.2 : 1 | 35.2 | 21.8 | 1.6:1 |
| 1914 | | | | | | | | |
| Jan. 19 | Ontario fine sandy loam | 10,000,000 | 7,000,000 | 3,000,000 | 1.4 : 1 | 37.0 | 31.3 | 1.2:1 |
| Feb. 27 | Dunkirk fine sand | 9,000,000 | 2,400,000 | 6,600,000 | 3.8 : 1 | 15.8 | 2.2 | 7.2:1 |
| Mar. 14 | Dunkirk fine sand | 3,500,000 | 3,200,000 | 300,000 | 1.1 : 1 | 35.7 | 12.2 | 2.9:1 |
| May 9 | Dunkirk fine sand | 3,300,000 | 2,800,000 | 500,000 | 1.2 : 1 | 33.0 | 22.5 | 1.5:1 |
| May 18 | Ontario fine sandy loam | 6,400,000 | 5,800,000 | 600,000 | 1.1 : 1 | 32.0 | 21.0 | 1.5:1 |
| May 19 | Dunkirk sandy loam | 1,800,000 | 3,200,000 | −1,400,000 | 1 : 1.8 | 36.0 | 35.5 | 1:1 |
| May 21 | Muck | 11,000,000 | 10,000,000 | 1,000,000 | 1.1 : 1 | 14.5 | 19.5 | 1:1.3 |
| May 22 | Honeoye stony loam | 3,200,000 | 2,800,000 | 400,000 | 1.1 : 1 | 32.0 | 18.8 | 1.7:1 |
| May 28 | Ontario fine sandy loam | 10,500,000 | 4,300,000 | 6,200,000 | 2.4 : 1 | 36.8 | 20.0 | 1.8:1 |
| May 29 | Dunkirk silty clay loam | 8,000,000 | 4,000,000 | 4,000,000 | 2 : 1 | 40.0 | 19.0 | 2.1:1 |
| Aug 8 | Ontario loam | 6,000,000 | 6,400,000 | −400,000 | 1 : 1.1 | 28.5 | 25.5 | 1.1:1 |
| Aug. 27 | Dunkirk silty clay loam | 9,600,000 | 1,500,000 | 8,100,000 | 6.4 : 1 | 60.0 | 14.8 | 4:1 |
| Aug. 28 | Ontario loam | 10,300,000 | 9,000,000 | 1,300,000 | 1.1 : 1 | 44.5 | 28.8 | 1.5:1 |
| Sept. 1 | Dunkirk silty clay loam | 12,000,000 | 2,400,000 | 9,600,000 | 5 : 1 | 34.4 | 14.0 | 2.4:1 |
| Sept. 2 | Dunkirk silty clay loam | 7,000,000 | 2,200,000 | 4,800,000 | 3.2 : 1 | 58.0 | 19.0 | 3:1 |
| Sept. 10 | Dunkirk silty clay loam | 8,500,000 | 2,500,000 | 6,000,000 | 3.4 : 1 | 47.2 | 13.8 | 3.4:1 |
| Sept. 11 | Ontario fine sandy loam | 4,500,000 | 1,600,000 | 2,900,000 | 2.8 : 1 | 45.0 | 16.0 | 2.8:1 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Oct. 23 | Dunkirk silty clay loam | 8,500,000 | 3,000,000 | 5,500,000 | 2.8 : 1 | 38.6 | 10.0 | 3.9:1 |
| Oct. 30 | Dunkirk silty clay loam | 4,700,000 | 2,700,000 | 2,000,000 | 1.7 : 1 | 42.8 | 15.9 | 2.7:1 |
| 1915 | | | | | | | | |
| Sept. 8 | Dunkirk silty clay loam | 7,500,000 | 4,500,000 | 3,000,000 | 1.7 : 1 | 64.0 | 23.0 | 2.8:1 |
| Sept. 16 | Dunkirk silty clay loam | 12,000,000 | 2,500,000 | 9,500,000 | 4.8 : 1 | 48.5 | 12.5 | 3.9:1 |
| Sept. 21 | Dunkirk fine sand | 1,600,000 | 1,300,000 | 300,000 | 1.2 : 1 | 27.5 | 22.0 | 1.3:1 |
| Sept. 24 | Ontario fine sandy loam | 7,000,000 | 4,600,000 | 2,400,000 | 1.5 : 1 | 30.0 | 20.0 | 1.5:1 |
| Oct. 11 | Dunkirk sandy loam | 2,200,000 | 630,000 | 1,600,000 | 3.5 : 1 | 32.5 | 17.5 | 1.9:1 |
| Oct. 20 | Homeoye stony loam | 8,000,000 | 5,000,000 | 3,000,000 | 1.6 : 1 | 30.8 | 19.5 | 1.6:1 |
| Oct. 22 | Dunkirk gravelly loam | 13,500,000 | 3,500,000 | 10,000,000 | 3.8 : 1 | 46.0 | 17.0 | 2.7:1 |
| Nov. 4 | Dunkirk fine sand | 2,800,000 | 2,800,000 | 0 | 1 : 1 | 35.0 | 3.8 | 1:1 |
| Nov. 4 | Honeoye stony loam | 6,000,000 | 6,300,000 | −300,000 | 1:1.05 | 21.5 | 25.0 | 1:1.2 |
| Nov. 4 | Dunkirk sandy loam | 1,300,000 | 2,400,000 | −1,100,000 | 1:1.8 | 29.0 | 36.5 | 1:1.3 |
| Nov. 4 | Dunkirk gravelly loam | 9,500,000 | 5,500,000 | 4,000,000 | 1.7 : 1 | 46.0 | 18.5 | 2.5:1 |
| 1916 | | | | | | | | |
| Jan. 27 | *Dunkirk fine sand | 1,600,000 | 1,200,000 | 400,000 | 1.3 : 1 | 45.0 | 30.0 | 1.5:1 |
| Jan. 28 | *Honeoye stony loam | 6,600,000 | 5,400,000 | 1,200,000 | 1.2 : 1 | 36.5 | 35.5 | 1:1 |
| Jan. 28 | *Dunkirk sandy loam | 1,800,000 | 1,600,000 | 200,000 | 1.1 : 1 | 60.0 | 52.0 | 1.2:1 |
| Jan. 29 | *Dunkirk gravelly loam | 12,800,000 | 10,500,000 | 2,300,000 | 1.2 : 1 | 51.0 | 26.5 | 1.9:1 |
| Average | | 6,850,000 | 3,800,000 | 3,000,000 | 2.15:1 | 37.5 | 20.5 | 2.1:1 |

* The last four pairs of analyses were made from the same samples as those on Nov. 4, 1915. During the intervening twelve weeks they had been kept in the laboratory. Meanwhile the Dunkirk fine sand and sandy loam had become almost completely dry. As these later analyses did not represent normal field conditions, their results are omitted from the averages in this table.

In making a comparison of these ratios it seemed reasonable to assume that those falling between the limits of 1.2: 1 and 1:1.2 were so near unity as to indicate no real difference in numbers between the sod and cultivated samples. In the sixth column, giving the ratios of the actual numbers per gram, sixteen cases are listed that fell within these limits. There were only two cases (May 19, 1914 and the third on November 4, 1915) when the numbers in cultivated soil were enough greater than in sod to give a ratio outside these limits; and of these the greatest ratio was only 1: 1.8. There were nineteen cases, however, in which the numbers in sod were sufficiently greater than in cultivated soil to give a ratio exceding 1.2: 1; and of them the maximum ratio was 6.4: 1. The average ratio of all thirty-eight cases was 2.15: 1, which is larger than the ratio between the general averages of columns three and four. The figures which show what percentage of the total flora consisted of *Actinomycetes* are somewhat more striking. The average ratio, it is true, (as shown in the last column of the table) was 2.1: 1 or practically the same as the ratio between the actual numbers per gram; but there were only four cases that fell between the limits 1.2: 1 and 1: 1.2 and only two (May 21, 1914 and the third on November 4, 1915) when the numbers in cultivated soil were enough greater than in sod to give a ratio outside these limits. These two cases both showed a ratio of 1: 1.3 which is hardly to be compared with the maximum ratio, 7.2: 1, in favor of sod soil.

The conclusion to be drawn from this comparison is that the few exceptional cases in which there were more *Actinomycetes* in the cultivated soil are completely overbalanced by the numerous cases in which there were more in the sod soil. In some of the border-line cases, moreover, the number of lower bacteria was greater in the cultivated soil than in the corresponding sod sample, with the result that the percentage of *Actinomycetes* was sometimes greater in the sod sample even though the actual number was the same in both samples.

The last four cases in the table are of special interest because they were analyses of the same samples collected on November

4, 1915, made after keeping the samples in the laboratory twelve weeks. On the date of collection the ratio obtained in the case of one pair of samples was in favor of the cultivated soil, while in two of the others case it was nearly unity. At the time of the later analysis the ratios in these three cases were still all near unity, although none of them were actually in favor of cultivated soil.

The results of the other series of tests, comparing three neighboring spots in a single soil type, are given in Table III. The

TABLE III

*Number of Actinomycetes in three neighboring spots of a single soil type. A comparison of old sod, new sod, and cultivated soil. Numbers determined by means of gelatin plates*

| DATE | ACTUAL NUMBER PER GRAM | | | PER CENT OF TOTAL FLORA | | |
|---|---|---|---|---|---|---|
| | Old sod | New sod | Cultivated | Old sod | New sod | Cultivated |
| May 29, 1914... | 8,000,000 | | *4,000,000 | 40.0 | | *19.0 |
| September 1, 1914... | 12,000,000 | | *2,400,000 | 34.4 | | *14.0 |
| September 5, 1914... | | 5,000,000 | 3,000,000 | | 20.8 | 15.2 |
| September 10, 1914... | 8,500,000 | 7,500,000 | 2,500,000 | 47.2 | 21.7 | 13.8 |
| October 23, 1914... | 8,500,000 | 7,800,000 | 3,000,000 | 38.6 | 25.2 | 10.0 |
| September 16, 1915... | 12,000,000 | 6,600,000 | 2,500,000 | 48.5 | 23.0 | 12.5 |
| Average..... | 9,800,000 | 6,600,000 | 2,900,000 | 41.7 | 23.6 | 14.1 |

* The first two samples of cultivated soil were taken from a different spot from the rest, although similar in kind of soil and in state of cultivation.

numbers obtained in this test were so constant that the few analyses mean as much as longer series of irregular results. The average number of *Actinomycetes* in the old sod was 9,800,000 per gram, in the new sod 6,600,000 and in the cultivated soil, 2,900,000; or in percentages, they averaged 41.7, 23.6 and 14.1 per cent, respectively, of the total flora in these three spots. The lowest count (of *Actinomycetes*) in old sod was higher than the highest in new sod, and the lowest in new sod higher than the highest in cultivated soil. These figures indicate that the number of *Actinomycetes* in sod soil increases as the age of the sod grows greater.

The interpretation of the figures hinges upon the question whether these organisms should be regarded as filametous fungi producing spores or as unicellular bacteria occurring in filaments. On ordinary culture media they exist as branched filaments that break up under certain conditions into short rods or coccus-like bodies, known as conidia because of their similarity to the conidia of molds in method of formation. When such cultures are plated, each colony ordinarily comes from one conidium or group of conidia. If they grow similarly in the soil and if the conidia are actually spores, an increase in the number of colonies on the plates may indicate merely an increase in spore-production. A few observations are at hand, however, to indicate that *Actinomycetes* occur in the soil not as filaments but as chains of short rods or cocci closely resembling ordinary bacteria. If this is the normal mode of growth in the soil and if these bodies are individuals instead of spores, an increase in the number of colonies on the plates may be regarded as more nearly representing a true increase in the number of the organisms in the soil.

Making the assumption that the latter condition actually exists in the soil, which seems justified so far as the facts are known, there are two explanations of the higher numbers observed in sod soil that seem sufficiently probable to be considered. One is that sod soil becomes more compact in time than cultivated soil and that poor aeration favors the *Actinomycetes* in some way, in spite of the fact that they ordinarily seem to like a good supply of oxygen. This explanation does not well fit the facts, however; for it has been found that sod soil, dug up and well aerated and then kept in a pile for three months, may still retain its high *Actinomyces* content. The other explanation which has been considered is that the *Actinomycetes* are active in the decomposition of grass roots or perhaps of plant roots in general. In view of the past observations as to the association between *Actinomycetes* and plant roots, this explanation seems worth bearing in mind. Experiments are now being carried on which are designed to show whether or not this is the true function of *Actinomycetes* in soil.

SUMMARY

1. In general more colonies of *Actinomycetes* develop on plates made from sod soil than on those from cultivated soil. The average ratio between their numbers in neighboring sod and cultivated spots in the same soil type is slightly over 2: 1. The maximum ratio is about 6: 1.

2. *Actinomycetes* average about 38 per cent of the total flora of sod soil, as determined by means of gelatin plates, but only about 20 per cent of the total flora of cultivated soil.

3. In a study of three neighboring spots in a single soil type it has been found that *Actinomyces* colonies not only appear in greater numbers from sod than from cultivated soil, but also in greater numbers from old sod than from sod only two or three years old.

4. This relation has been found to hold with very few exceptions In the isolated cases where more *Actinomyces* colonies have developed from a sample of cultivated soil than from the corresponding sample of sod soil, the ratio has never been greater than 1.8: 1.

5. Although the reason for this difference in numbers has not been learned, a probable explanation seems to be that *Actinomycetes* are active in the decomposition of grass roots.

BIBLIOGRAPHY

BEIJERINCK, M. W. 1900. Ueber Chinonbildung durch Streptothrix chromogena und Lebensweise dieses Mikroben. Centbl. f. Bakt. Abt. 2, *6*, 2–12.

GASPERINI, G. 1894. Versuche über das Genus "Actinomyces." Paper presented at the Eleventh International Medical Congress at Rome. Abstract, Centbl. f. Bakt. Abt. 1, *15*, 684.

HILTNER, L. AND STÖRMER, K. 1903. Studien über die Bakterienflora des Ackerbodens, mit besonderer Berücksichtigung ihres Verhaltens nach einer Behandlung mit Schwefelkohlenstoff und nach Brache. Kaiserliches Gesundheitsamt, Biol. Abt. Land- u. Forstw. *3*, 445–545.

KRAINSKY, A. 1914. Die Aktinomyceten und ihre Bedeutung in der Natur. Centbl. f. Bakt., Abt. 2, *41*, 649–688.

LUTMAN, B. F. AND CUNNINGHAM, G. C. 1914. Potato Scab. Vermont Agric. Exper. Sta., Bul. 184.

# PRACTICAL OBSERVATIONS ON THE TITRATION AND ADJUSTMENT OF CULTURE MEDIA

BERTHA VAN HOUTEN ANTHONY AND CLARENCE V. EKROTH

*Bureau of Laboratories, Department of Health, City of New York*

Any one who studies the methods given in the various textbooks for the titration and adjustment of culture media, must be struck by the lack of uniformity of opinion. Not only is the beginner in media preparation bewildered but even the more experienced worker may be led into error. The difficulty arises from the fact that the complex nature of the materials dealt with is by no means fully understood, even in the case of the most fundamental culture media. In addition to this the changes that occur under even slightly different conditions and treatment are most confusing. As a result each laboratory is compelled to adopt the methods best adapted to its work and requirements, and each laboratory makes changes in these methods as need arises.

The many requests constantly made for information regarding the methods employed in our laboratories for the titration and adjustment of both general and certain special culture media seem to indicate the need for a detailed account of such procedures.

In this paper we have tried to incorporate the practical information gained after a number of years of experience. In addition, we have described experiments carried on with a view of clearing up, in a systematic manner, certain points upon which little, if any, information is available.

The *Standard Method*[1] of titrating media for water and milk analyses was devised in an attempt to secure uniform preparations of media at all times so that comparable results might be obtained.

[1] Committee on Standard Methods, 1905, 1913).

The directions are as follows:

Phenolphthalein shall be the standard indicator in obtaining reaction of all media. Tumeric paper possesses similar properties and its use advised where phenolphthalein is not available. Titrations and adjustments of reactions shall be made as follows:

Put 5 cc. of media to be titrated in 45 cc. of distilled water. Boil briskly one minute. Add 1 cc. phenolphthalein solution (5 grams of commercial salt in one liter of 50 per cent alcohol.) Titrate while hot (preferably while boiling) with $\frac{N}{10}$ caustic soda. A faint but distinct pink marks the true end point. This distinct pink color may be described as a combination of 25 per cent of red (wave length approximately 658) with 75 per cent of white as shown by the disks of the color top, described under Records of Tints and Shades of Color, p. 10. (The Standard[2] color disks used in teaching optics may be used for this purpose.)

In practice, titration is continued until the pink color of alkaline phenolphthalein matches that of the fused disks. All reactions shall be expressed with reference to the phenolphthalein neutral point and be expressed in percentages of normal acid or alkaline solutions required to neutralize them.

One of the objects of this paper is to consider whether the desired results are actually obtained by the Standard method or by modifications of this method.

In our laboratory one modification, that is, titration of broth at room temperature (about 20°C.) and of agar at a temperature of about 30°C. has given good results for a number of years past.

These and other experiences have led us to investigate further the following subjects:

*a.* The effect of prolonged heating on meat infusions and beef extract solutions as shown by the titration curves of both adjusted and unadjusted portions. The results of boiling samples of media in the casserole for titration.

*b.* The adjustment of broth and agar, including the remelting of solid media.

[2] A small sized top and disks costing only a few cents may be obtained from Milton Bradley Educational Company, Springfield, Mass.

*c.* The reaction of peptone solutions and the effects upon them of prolonged heating.

*d.* The question of indicators with consideration of the significance and sensitiveness of their end points in different media—also the method of choosing the one most suitable with reference to the hydrogen electrode as a standard.

## MEAT INFUSIONS

It is a well known fact that each time a medium is heated to the boiling point, or above it, the reaction changes and becomes more and more acid, depending on the length of time and the degree of heating. On this subject Eyre (1915) says:

> Meat extract [meat infusion] is acid in reaction owing to presence of acid phosphates of potassium and sodium; weak acids of the glycolic series and organic compounds in which an acid character predominates.
>
> Owing to the nature of the substances from which it derives its reaction, the total acidity of meat extracts [infusion] can only be estimated accurately when the solution is at the boiling point. Prolonged boiling [as in media preparation] causes it to undergo hydrolytic changes which increase the acidity.

He states further that meat extract [infusion] becomes stable in reaction after being heated at the boiling point for forty-five minutes so that no additional increase of acidity occurs on further heating.

To procure more definite data as to the effect of heat on the acidity of media the following work was carried out:

*Preparation of the meat infusions.* Chopped lean veal was soaked over night in tap[3] water in the proportion of one pound of meat to one liter of water. It was then heated at 45° to 55° C. for one hour. At this point it was brought to a boil. Then

[3] Weekly analyses of the Croton water supply shows it to contain a negligible amount of mineral matter (only about 40 parts per million, expressed as total hardness.) Where the water supply is at all "hard," it is advisable to employ distilled water exclusively.

the material (meat and watery infusion) was divided into three lots:

B[4] 30 was kept at the boiling point 30 minutes.
B 60 was kept at the boiling point 60 minutes.
B 120 was kept at the boiling point 120 minutes.
(Volumes being made up by addition of tap water.)

After being boiled, each lot was strained through cheesecloth, then filtered through paper (S. & S. "Falten" filter) and cotton (first moistened with cold water to hold back the fatty substances.)

Each lot was titrated[5] and then divided again into two parts and one series (B 30, B 60, B 120) was run in the autoclave at least six successive times, without the addition of soda.

The second series (B 30 C, B 60 C, B 120 C) was corrected with normal sodium hydroxid to 1 per cent acidity (+1) and then run in the autoclave with the other set and under the same conditions.

In all the tests made the autoclaving was done at a pressure between 15 and 17 pounds as indicated by a Bristol recording pressure gauge. The heating was carried on up to a total of eight hours and titrations performed at one-half hour intervals for the first four half-hours; then at one hour, two hour and three hour intervals.

After each autoclaving the six samples were titrated and the corrected series (B 30 C, B 60 C, etc.) was adjusted again when necessary to plus one (+ 1).

*Method of titration.* Freshly boiled and cooled distilled water was used for all titrations. A 5 cc. sample of meat infusion was drawn off by means of a 5 cc. pipette and added to 45 cc. of distilled water in a casserole to which 1 cc. of a 1 per cent[6] solution

[4] Preliminary titrations on samples before boiling had been labelled "A." As these had no significance they are omitted in this article.

[5] See: "Method of Titration."

[6] These are also variations from the standard method of using 0.5 per cent solution of phenolphthalein and twentieth normal sodium hydroxid solution. They are, however, in accordance with the methods in use for years in this laboratory and to preserve uniformity they were adhered to.

of phenolphthalein had been added. While stirring the mixture, deci-normal[6] soda solution from a burette was run in without any heating whatever. The end point taken was the first delicate pink tinge, observable throughout, which did not disappear after stirring the solution—and should not disappear for at least one minute. The figures were then recorded. The casserole with the mixture was then set over the flame, brought to a boil and boiled one minute by the watch.

*Outline of Experiments*

*Meat infusions* First lot of veal

- B 30 = preliminary boiling of 30 min.
- B 60 = preliminary boiling of 60 min.
- B 120 = preliminary boiling of 120 min.
- B 30 C = preliminary boiling of 30 min.
- B 60 C = preliminary boiling of 60 min.
- B 120 C = preliminary boiling of 120 min.

Corrected to plus one according to *room temperature* titration after each autoclaving.

- $B_1$ 60 = preliminary boiling of 60 min.
- $B_1$ 120 = preliminary boiling of 120 min.
- $B_1$ 60 C = preliminary boiling of 60 min.
- $B_1$ 120 C = preliminary boiling of 120 min.

Corrected to plus one according to *boiling temperature* titration after the *first* autoclaving. No further adjustments were made because of error in adding too much soda.

*Meat infusions* Second lot of veal

- $B_2$ 30 = preliminary boiling of 30 min.
- $B_2$ 60 = preliminary boiling of 60 min.
- $B_2$ 120 = preliminary boiling of 120 min.
- $B_2$ 30 C = preliminary boiling of 30 min.
- $B_2$ 60 C = preliminary boiling of 60 min.
- $B_2$ 120 C = preliminary boiling of 120 min.

Corrected to plus one according to *boiling temperature* titration after each autoclaving.

Since the boiling had caused the faint pink color to disappear, the hot mixture was then promptly titrated again, the same end point being approximated[7] as closely as possible, and the figures recorded. The room temperature figure plus that obtained after boiling one minute gave a total which represents the boiling titration figure as recorded in the charts.

In the first lot of veal, (series B 30, B 60, B 120), the corrections were made to plus one at the room temperature figures. A second lot of veal infusions ($B_2$ 30, $B_2$ 60, $B_2$ 120) were prepared in the same manner as above. The corrections in this lot were made to plus one at the boiling figures.

## DESCRIPTION OF CHARTS

In charts 1 and 2 are shown the uncorrected portions of meat infusions titrated after successive heatings in the autoclave and plotted according to both the room temperature and the boiling temperature figures. It will be seen that in each lot of meat the room temperature titrations fall into one group and the boiling titrations into another; also that the boiling figures are the higher.

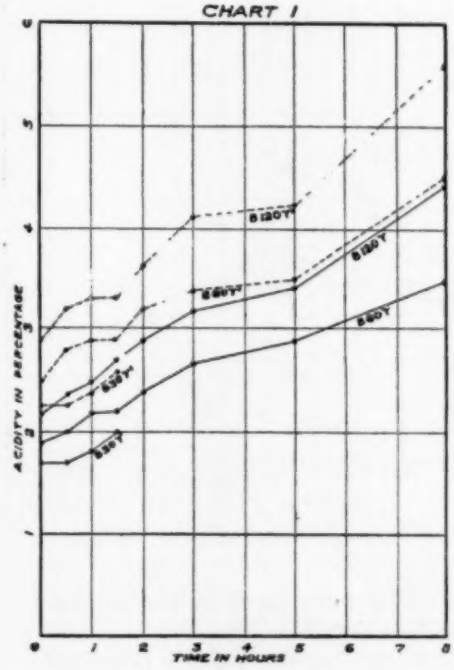
Chart 3 is a sample[8] chart showing not only the uncorrected portion of B 120 as given in Chart 1, but also the corrected portion, B 120 C. This portion was corrected to plus one (+ 1) according to the room temperature titration figures and readjusted to plus one after each autoclaving, as shown by the $\alpha$ line. The $\alpha'$ line shows the figures of this same material when boiled one minute in the casserole and titrated hot. This line is hypothetical and shows only the amount of soda that would have been needed had the boiling figures been used for adjustment to plus one.

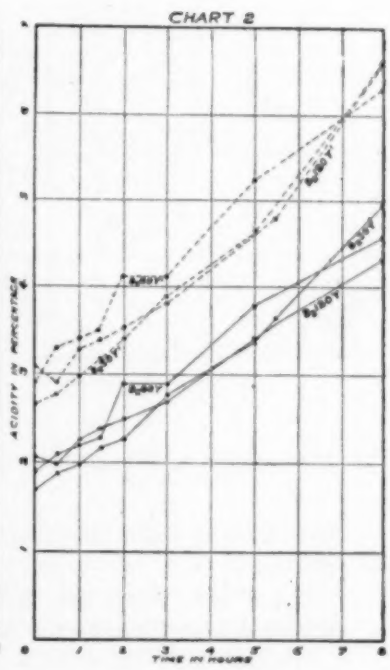
The $\theta$ line shows the total amount of acidity produced in the corrected portion even after the addition of soda, according to the room temperature figures.

[7] The difficulty of catching the first color change of phenolphthalein in hot solutions will be discussed under "Indicators."

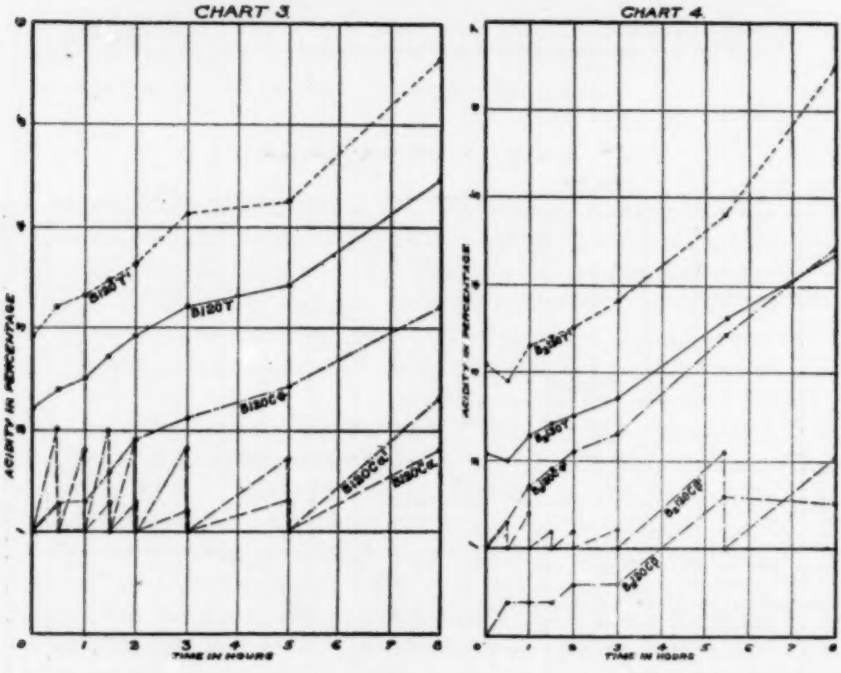
[8] This chart is typical also of the B 30 and B 60 sets.

| KEY TO CURVE NOTATION. | | |
|---|---|---|
| LETTER | LINE | DENOTES |
| $\alpha$ | ——— | = corrected at room temperature |
| $\alpha'$ | ——·——·—— | = " " " "(boiling figures) |
| $\beta'$ | ——— ——·——— | = " " boiling temperature |
| $\beta$ | ——·——·——·—— | = " " "(room temp. figures) |
| $\gamma$ | ——————— | = uncorrected (room temperature) |
| $\gamma'$ | - - - - - - - - - - - | = " (boiling temperature) |
| $\phi$ | ——·——·—— | = Total acidity, corrected at room temp. |
| $\phi'$ | ——··——··—— | = " " " " " boiling temp. |



In Chart 4[9] is represented $B_2$ 120 (second lot of veal) just as B 120 is shown in Chart 3 except that here the corrected portion ($B_2$ 120 C$\beta'$) was adjusted to plus one (+1) according to the boiling titration figures. The $\beta$ line shows the figures obtained each time at room temperature before the sample was boiled one minute in the casserole to give the boiling titration




figures. The room temperature curve is inserted here for comparison.

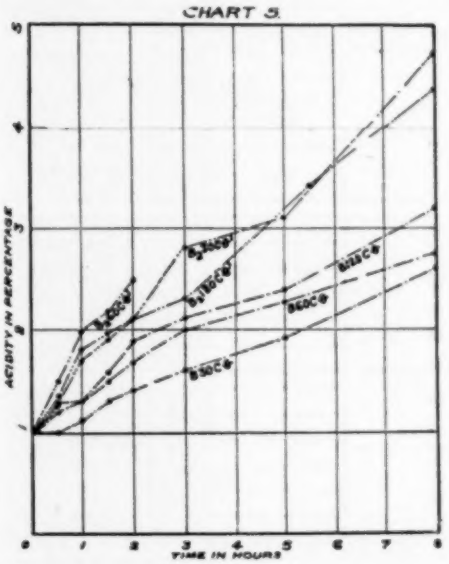
The $\theta'$ line shows the total acidity produced in the portion corrected according to the boiling titration figures.

In chart 5 all the total acidity lines of the various corrected portions are compared. As with the uncorrected portions as

[9] This chart is typical also of the $B_2$ 30 and $B_2$60 sets.

noted in charts 1 and 2, the total acidity lines fall into two groups according to the method of titration.

If chart 5, showing the total rises in acidity of the series B 30 C, B 60 C, B 120 C and the series $B_2$ 30 C, $B_2$ 60 C, $B_2$ 120 C, were applied successively to the corresponding curves of the uncorrected series in charts 1 and 2, so that the point of origin in each set were the same, it would be seen that in every instance the

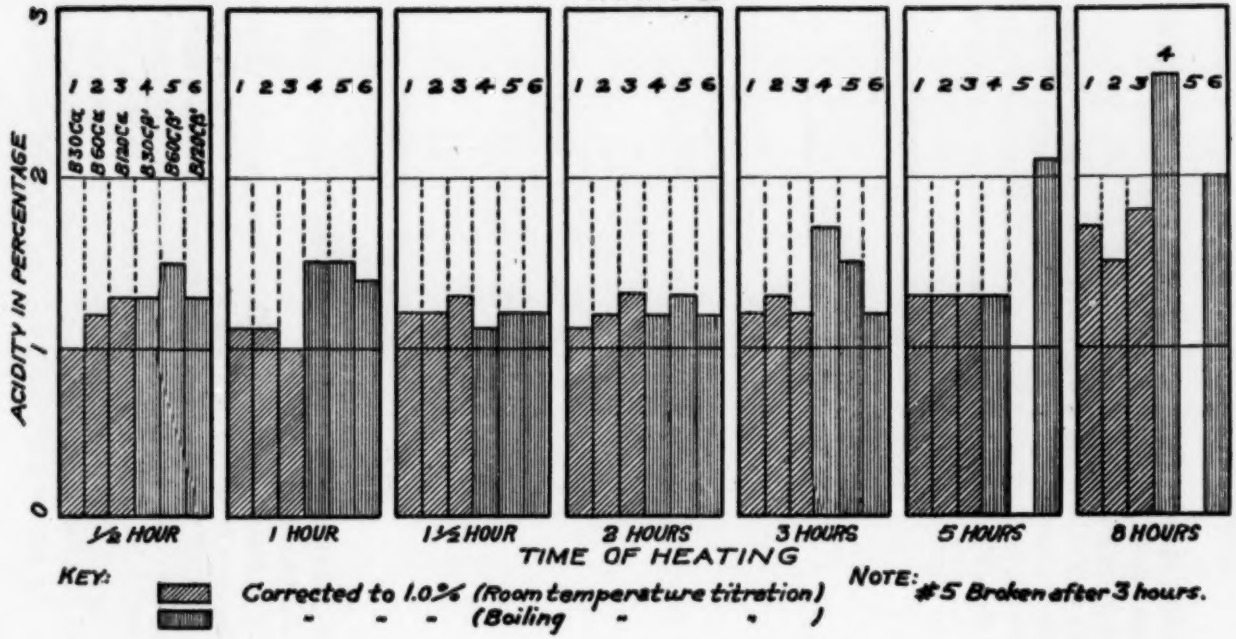



total rise of acidity of the corrected curve equals or exceeds that of the uncorrected portion.

From this it is plainly evident that, in spite of successive corrections of acidity with normal sodium hydroxid, hydrolysis not only continues on the application of heat but there is produced in meat infusion media approximately as much acidity as would be developed were no correction made.

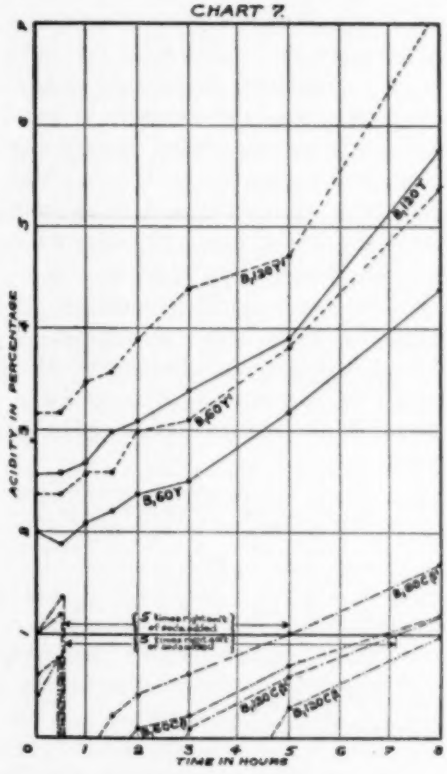
Chart 6 shows the actual acidity of the different corrected portions after the successive adjustments and periods of heating. As can be seen, those portions adjusted according to the

## CHART 6.


ACIDITY IN PERCENTAGE
0
1
2
3
1 2 3 4 5 6
830Cα
860Cα
8120Cα
830Cβ'
860Cβ'
8120Cβ'
½ HOUR
1 HOUR
1½ HOUR
2 HOURS
3 HOURS
5 HOURS
8 HOURS
TIME OF HEATING
KEY:
Corrected to 1.0% (Room temperature titration)
(Boiling - - - )
NOTE: #5 Broken after 3 hours.

room temperature titration are in general nearer to the desired reaction of plus one (+ 1), especially in the first two half-hours—the periods of time of greatest practical interest.

In chart 7 are shown not only the uncorrected portions of $B_1$ 60 ($\gamma$ and $\gamma'$) and $B_1$ 120 ($\gamma$ and $\gamma'$) (duplicate material from




first lot of veal), but also the effect on the corrected portions of the addition of too much normal sodium hydroxid. By mistake after the first half hour in the autoclave and the subsequent titration, there was added to $B_1$ 60 C $\beta'$ approximately five times the amount of normal sodium hydroxid necessary to correct it to plus one, and to $B_1$ 120 C $\beta'$ also about five times

the right amount. These amounts were in accordance with the boiling titration figures in both cases. The quantities needed for correction were, in round numbers, twice as much for the latter as for the former. The relation of the quantities of alkali added may, therefore, be expressed by the numerical relation of ten to five. The portions of meat infusion (without further additions of soda) were then run as usual in the autoclave with the uncorrected portions and titrated at the same intervals.

Curiosity led us to continue these tests rather than discard them. In consequence, an interesting fact was brought out. To our surprise both over-corrected portions recovered the acidity of plus one (and more) but at different intervals of time. $B_1$ 60 C$\beta'$ gained plus one at the end of the fifth hour while $B_1$ 120 $\beta'$ reached plus one at about the seventh hour.

In all the tests the curves show a distinct and steady rise in acidity. This rise is due to hydrolysis caused by heat and increases continually as more heat[10] is applied. Further, it is plainly evident that a preliminary heating to the boiling point for at least forty-five minutes as advocated by Eyre (1915) does not produce a stable reaction uninfluenced by further heating.

TABLE I

| MEAT INFUSION | REACTION BEFORE AUTOCLAVING | | AFTER 8 HOURS IN AUTOCLAVE | | AFTER 14 HOURS IN AUTOCLAVE | |
|---|---|---|---|---|---|---|
| | R. T.* | B. T.† | R. T. | B. T. | R. T. | B. T. |
| $B_1$30 | 1.7 | 2.7 | 4.9 | 6.5 | 7.4 | 10.5 |
| $B_1$60 | 1.9 | 2.9 | 4.6 | 6.3 | 7.3 | 10.4 |
| $B_1$120 | 2.1 | 3.1 | 4.3 | 6.5 | 6.9 | 9.3 |

*R. T. = room temperature titration.
*B. T. = boiling temperature titration.

In the above tests we have gone outside the limits of interest from the practical standpoint. We were led to this, however, in an attempt to locate the point of complete hydrolysis or maximum acidity of meat infusions. This goal was not reached,

[10] Three of the uncorrected portions, $B_1$ 30, $B_1$ 60, $B_1$ 120 were run an additional six hours, making fourteen hours all told in the autoclave at 15 pounds pressure. The results are shown in last columns of Table I.

as stated above, even after fourteen hours autoclaving. We are continuing this work.

In the usual preparation of media from meat the total amount of heating in the autoclave varies from one half hour, at about 15 pounds pressure, for sterilization of ordinary broth, to two and one-half hours in the preparation and sterilization of agar.

It developed in the tests made on the corrected meat infusions that the change in acidity in the first half hour in the autoclave (the usual time for the sterilization of finished media) varied from nothing to 0.3 per cent at the room temperature figures, while in boiling temperature figures the change in acidity was 0.3 to 0.4 per cent (see chart 6).

The nature of the acid products which are formed as a result of the hydrolytic decomposition on boiling with sodium hydroxid may be different from those produced by hydrolysis alone when boiling unadjusted media. Therefore, while the reaction may be adjusted in each to the same point of acidity, the behavior of the media toward the various organisms may not be the same.

Similar tests to those above were made on Liebig's beef extract dissolved in tap water, filtered and titrated in a similar fashion. These tests showed no change in the reaction of the uncorrected series even after eight hours heating. Although a beef extract solution is undoubtedly quite stable,[11] as compared with meat infusions, a sample corrected[12] to neutral, after two hours in the autoclave, rose 0.2 per cent in acidity. This was corrected to neutral once more and did not change again although heated four hours longer.

While the above change is almost negligible, the addition of peptone to this as to other media, raises the change in acidity further. This must be taken into consideration in the preparation of media with beef extract when delicate end points are desired.

[11] This stability is due probably to very prolonged heating in the preparation of the beef extract itself.

[12] Correction based on room temperature titration.

## BROTH[13]

The tests on the meat infusions were carried out before any peptone or salt had been added. In the preparation of broth the choice of titration methods must of course be governed by the manner of preparing the meat juice in the preliminary steps. The relative merits of pressing out the meat juice before or after heating the soaked meat must be determined by experiment in the kinds of work for which the media are destined.

For those workers whose needs and experience lead them to express the meat juice in the cold state and then dissolve the peptone and salt with very little preliminary heating, the use of the boiling titration for correction is essential, in order to approximate the future conditions due to further heating and sterilization after the reaction of the batch of medium has been set.

In this laboratory the best toxin production has seemed to be obtained when the meat juice is pressed out after heating large amounts (20 liters) for one hour at 45° to 55°C. and then boiling up strongly until the meat coagulates. The meat infusion[14] is then strained through cheese-cloth. After the requisite amount of peptone and salt have been dissolved by further heating up to the boiling point and the mixture boiled one half hour, the reaction is set according to a room temperature titration.

If the specimen is boiled[15] in the casserole before titration, it no longer represents the lot of broth in the kettle but has risen somewhat in acidity. Consequently, if the large lot be adjusted according to the boiling titration a false correction is made. To be sure, after adding the normal soda solution,

[13] Broth = meat infusion plus peptone and, usually, salt.

[14] This method proves useful in other lines of work for at this point the infusion can be filtered, sterilized and stored. It is ready for further use on the addition of any suitable peptone and may be set at any desired reaction; or it is ready as a basis for making agar.

[15] At this point may be mentioned the length of time recommended for boiling by different authors. Heinemann (1911) heats "to boiling." Jordan (1914) MacNeal (1914) and the Standard Method (1913) boil one minute. Park and Williams (1914) boil two minutes, Abbott (1915), Abel (1914), Hiss and Zinsser (1914), Mallory and Wright (1915), Swithinbank and Newman (1903) boil for three minutes.

any further boiling of the large lot, together with the final sterilization raises the acidity but not to just the desired point as shown in the tests on meat infusions (chart 6). For example, the final reaction of + 1.2 in the case of diphtheria toxin broth, is found to be uniformly obtained by titrating at room temperature and setting the reaction to + 1. This allows 0.2 rise due to heating if the broth is to be sterilized at 15 pounds pressure (121.6°C.) for one half hour. If the sterilization is to be carried on at only 5 pounds pressure (108.8°C.) for one hour on three successive days, or in the Arnold sterilizer, streaming steam (100°C.), for the same length of time, the reaction is set at + 1.1 as the more moderate heating raises the broth only about 0.1 per cent in acidity, making the finished product + 1.2 in reaction. The use of this method for the diphtheria and tetanus toxin broths for a number of years has shown fully its value and it is the method still employed in our laboratory.

On the other hand Eyre (1915) states that the correct estimation of acidity present can be made only by titration at the boiling point. Judged from our results as shown in chart 4 by the $\beta'$ and $\beta$ curves, this statement is erroneous. The $\beta'$ curve shows the reactions and successive adjustments to plus one (+ 1) based upon the *boiling temperature* figures. The reactions at room temperature of this material are shown by the $\beta$ curve. This latter is far below the $\beta'$ curve (from 0.5 to 1 per cent), and gives the actual reaction of the medium at a temperature nearer that of the incubator (37.5°C.)[16]

To make this point clear, let us assume for example that a medium is to be adjusted to a definite acidity of + 1, according to the boiling titration, as stated in the Standard Method. The real reaction at which the bacteria will then be grown in the incubator is not that indicated by the boiling titration figure but a reaction which is lower in acidity to an extent of about one per cent—in other words, almost neutral.

On the other hand in chart 3 ($\alpha'$ curve), there is shown a similar meat infusion, adjusted to plus (+ 1) at *room temperature*

[16] It is only after four hours' autoclaving that the room temperature reaction of plus one (+1) is reached.

## BROTH[13]

The tests on the meat infusions were carried out before any peptone or salt had been added. In the preparation of broth the choice of titration methods must of course be governed by the manner of preparing the meat juice in the preliminary steps. The relative merits of pressing out the meat juice before or after heating the soaked meat must be determined by experiment in the kinds of work for which the media are destined.

For those workers whose needs and experience lead them to express the meat juice in the cold state and then dissolve the peptone and salt with very little preliminary heating, the use of the boiling titration for correction is essential, in order to approximate the future conditions due to further heating and sterilization after the reaction of the batch of medium has been set.

In this laboratory the best toxin production has seemed to be obtained when the meat juice is pressed out after heating large amounts (20 liters) for one hour at 45° to 55°C. and then boiling up strongly until the meat coagulates. The meat infusion[14] is then strained through cheese-cloth. After the requisite amount of peptone and salt have been dissolved by further heating up to the boiling point and the mixture boiled one half hour, the reaction is set according to a room temperature titration.

If the specimen is boiled[15] in the casserole before titration, it no longer represents the lot of broth in the kettle but has risen somewhat in acidity. Consequently, if the large lot be adjusted according to the boiling titration a false correction is made. To be sure, after adding the normal soda solution,

[13] Broth = meat infusion plus peptone and, usually, salt.

[14] This method proves useful in other lines of work for at this point the infusion can be filtered, sterilized and stored. It is ready for further use on the addition of any suitable peptone and may be set at any desired reaction; or it is ready as a basis for making agar.

[15] At this point may be mentioned the length of time recommended for boiling by different authors. Heinemann (1911) heats "to boiling." Jordan (1914) MacNeal (1914) and the Standard Method (1913) boil one minute. Park and Williams (1914) boil two minutes, Abbott (1915), Abel (1914), Hiss and Zinsser (1914), Mallory and Wright (1915), Swithinbank and Newman (1903) boil for three minutes.

any further boiling of the large lot, together with the final sterilization raises the acidity but not to just the desired point as shown in the tests on meat infusions (chart 6). For example, the final reaction of + 1.2 in the case of diphtheria toxin broth, is found to be uniformly obtained by titrating at room temperature and setting the reaction to + 1. This allows 0.2 rise due to heating if the broth is to be sterilized at 15 pounds pressure (121.6°C.) for one half hour. If the sterilization is to be carried on at only 5 pounds pressure (108.8°C.) for one hour on three successive days, or in the Arnold sterilizer, streaming steam (100°C.), for the same length of time, the reaction is set at + 1.1 as the more moderate heating raises the broth only about 0.1 per cent in acidity, making the finished product + 1.2 in reaction. The use of this method for the diphtheria and tetanus toxin broths for a number of years has shown fully its value and it is the method still employed in our laboratory.

On the other hand Eyre (1915) states that the correct estimation of acidity present can be made only by titration at the boiling point. Judged from our results as shown in chart 4 by the $\beta'$ and $\beta$ curves, this statement is erroneous. The $\beta'$ curve shows the reactions and successive adjustments to plus one (+ 1) based upon the *boiling temperature* figures. The reactions at room temperature of this material are shown by the $\beta$ curve. This latter is far below the $\beta'$ curve (from 0.5 to 1 per cent), and gives the actual reaction of the medium at a temperature nearer that of the incubator (37.5°C.)[16]

To make this point clear, let us assume for example that a medium is to be adjusted to a definite acidity of + 1, according to the boiling titration, as stated in the Standard Method. The real reaction at which the bacteria will then be grown in the incubator is not that indicated by the boiling titration figure but a reaction which is lower in acidity to an extent of about one per cent—in other words, almost neutral.

On the other hand in chart 3 ($\alpha'$ curve), there is shown a similar meat infusion, adjusted to plus (+ 1) at *room temperature*

[16] It is only after four hours' autoclaving that the room temperature reaction of plus one (+1) is reached.

*titration*. The boiling titration figures are plotted as curve $\alpha'$. The room temperature titration curve ($\alpha$) comes so near the desired reaction of plus one (+1) that even a mere glance will suffice to convince one that the room temperature titration approaches more closely the one per cent line, which is the acidity we set out to secure.

This was not surprising to us as our practical experience for several years past had indicated such a condition. The modification, (page 212), devised at that time and now further substantiated by these experiments, has proved to be so very useful in its results that it is employed in these laboratories for nearly all the routine preparation of some fifty different kinds of media, aggregating over 8000 liters per year.

## STERILE SODA

In order to avoid the complications of further hydrolysis and precipitation after the addition of soda to a medium which must later be sterilized, it has been suggested that sterilization be done first and that sterile soda be carefully added afterward according to the titration of samples withdrawn under sterile conditions. This has been practised by some workers, apparently with success. So far, in our laboratory, it has shown no advantages in the production of diphtheria toxin broth. Further work in this line is contemplated.

## AGAR

When the need arose of supplying large amounts of neutral veal agar for the growing of the gonococcus, streptococcus and other organisms in bulk for antigens, difficulty was experienced. To grow these organisms in large lots with unfailing success is not always easy. Our trouble seemed due chiefly to the reaction of the medium. Finally the modified titration method was adopted for agar also.

Since agar solidifies at a little below 40°C. the room temperature titration was not suitable. At first any temperature between

40° and 50°C. was used. This was reduced later to about 30°C.[17] Five cubic centimeters of the hot agar are added by means of a pipet to 45 cc. of distilled water—temperature about 30°C. (verified with a thermometer). One cubic centimeter of phenolphthalein (1 per cent solution in 50 per cent alcohol) is added and the titration performed at once.

It is now our custom to titrate each batch of neutral veal agar at least twice during its preparation making the necessary adjustments of reaction. Here, as with the broth, allowance must be made for further heating in the autoclave. Experience shows that agar made from meat infusion rises in acidity usually about 0.3 to 0.4 per cent. at 15 pounds pressure during one and one-half to two hours.[18] Therefore, 3 to 4 cc. of normal soda should be added per liter in excess of the amount required to secure the phenolphthalein neutral point at the time of the first titration. The second titration is made just after filtration, before tubing and sterilizing. If the amount of soda needed does not exceed 0.2 per cent, little if any precipitate occurs on heating further. If more than the above amount is needed in adjusting the reaction, the medium should be heated in the Arnold for half an hour and the precipitate filtered out, before tubing and sterilizing.

In very careful work the medium is also titrated a third time as it comes from the autoclave. For the last test a tube or small bottle of neutralized glassware should be used in order that the reaction of the agar may be unaffected by its container. This sample is tested before it hardens—as remelting would raise its acidity further. On the addition of phenolphthalein, it should show a very delicate shade of pink if it is "neutral."

### REMELTING OF SOLID MEDIA

An important factor to be considered in the adjustment of media is the remelting of solid media for the addition of sterile substances such as blood, serum, etc., or for the purpose of

[17] This slight difference of temperature had no noticeable effect on the results of the titration.

[18] This time is necessary for melting (and clearing with egg) of large batches of agar (5 to 12 liters).

immediate use in plating. If, for example, the whole is to be neutral to phenolphthalein when entirely finished an over neutralization is necessary to allow for the acid changes during the re-heating, as in making Bordet Gengou medium.

Since, in spite of the addition of soda for the correction of a medium further hydrolysis occurs when heat is applied, especially in the autoclave, it is impossible to know the exact reaction a medium will have when sterilization is complete or when the medium is re-melted. In practical work, however, it has been found that an over neutralization of 0.1 to 0.3 per cent has given good results when the titration is performed at 30°C.

The *re-sterilization* of media without suitable correction to allow for the effects of heating is to be avoided if a very definite end point is desired.

## PEPTONES

The present necessity of finding substitutes for Witte's peptone, so long the standard in bacteriological work, has led us to test the reaction of various peptones on the market. A 1 per cent solution of each in distilled water was boiled one minute and then filtered through cotton and filter paper. When cool, each was titrated at room temperature and then again after the same sample had been boiled one minute, that is, the same procedure was followed as in titrating the meat infusions (page 214).

The following table shows the reactions of eight peptones, including Witte's.

TABLE II

*Reaction of peptones (titrated with phenolphthalein)*

| KIND | ROOM TEMPERATURE FIGURE (AT 20 °C.) | RISE AFTER BEING BOILED ONE MINUTE IN CASSEROLE | BOILING TEMPERATURE FIGURE |
|---|---|---|---|
| Armour | +0.6 | +0.4 | +1.0 |
| Atkinson | +0.4 | +0.2 | +0.6 |
| Difco | +0.6 | +0.2 | +0.8 |
| Eimer & Amend | +1.0 | +0.4 | +1.4 |
| Fairchild culture | +0.7 | +0.4 | +1.1 |
| Leitz | +0.4 | +0.3 | +0.7 |
| Squibb | +0.3 | +0.1 | +0.4 |
| Witte | +0.3 | +0.1 | +0.4 |

The peptone solutions were then divided into two sets (as with the meat infusions), one corrected and the other uncorrected. These were given successive treatments in the autoclave and titrated at the same intervals. As with the meat infusions there was a steady increase in acidity though not in so marked a degree. Three uncorrected portions of peptone solutions (Fairchild's, Eimer and Amend, Squibbs) were also run an additional six hours, making fourteen hours in all in the autoclave. As with the meat infusions the limit of hydrolysis was not reached.

In the corrected portions the total amount of acidity, developed by heating after successive additions of normal soda, again paralleled closely the rise in acidity of the corresponding uncorrected portions.

From the above it is apparent that the introduction of a peptone into a medium will affect the reaction to some extent.

## INDICATORS

The shade of phenolphthalein suitable for a correct end point varies greatly in the opinion of different authors.

Miur and Ritchie (1913) give "the first trace of pink."

Hiss and Zinsser (1914)—"faint but clear and distinct pink."

Stitt (1913)—"a delicate pink (hot titration) a purplish violet color (cold titration)."

Jordan (1914)—"Faint but distinct pink color."

Park and Williams (1914)—"Faint, but distinct pink which remains on re-heating."

Heinemann (1911)—"Faint but decided and stable pink."

Abel (1912)—"Brilliant red." (Translation.)

Abbott (1915)—"Pink color."

MacNeal (1914)—"Faint but distinct and permanent pink."

Swithinbank and Newman (1903)—"Clear bright pink color."

Mallory and Wright (1915)—"Bright pink color" not "the pinkish darkening of the fluid which preceeds it."

Eyre (1915) uses a "pinkish tinge" or "a faint rose-pink which cooled to 30° or 20°C., becomes more distinct and decidedly deeper and brighter" resembling a "deep magenta color."

These shades given differ more or less from the Standard Method (1913).

That no two people seem to titrate to exactly the same shade has often been shown in our laboratory when a different worker in the media room has attempted to set the final reaction of some special medium. If the method of titrating is to be at all accurate, it is necessary to *assume* a shade of pink for phenolphthalein. This necessity is brought out by such great discrepancies between different workers titrating the same substance as are given by Clark (1915).[19] As a help, a practical color scale may be of aid in determining the most suitable shade for a certain kind of work and approximating it as closely as possible. This should eliminate the personal factor to some extent.

As stated before, in our opinion, the correct shade for a delicate end point in pale broth or other solutions with little color is the first most delicate pink tinge observable throughout, remaining at least one minute.

With us, when titrating agar, a mixture of 5/20 red, 3/20 orange and 12/20 white on the color-top (see page 210) has proved to be a desirable shade for the first and second titrations of neutral agar;[20] while the third titration, when the medium comes out of the autoclave, should give (on the addition of phenolphthalein) a shade consisting of 3/20 red, 3/20 orange and 14/20 white on the color-top. These shades differ from the ones given above both in the Standard Method of titrating at boiling point (page 210) and our own definition (page 213) but they have yielded very good results. However, it may be as difficult to decide on an end point by means of a color-top or scale as to imagine an end point from the descriptions of the various writers.

### LITMUS

Since any medium with meat infusion as a basis and peptone added is a most complex mixture, no one indicator shows all

[19] (16) page 117. Such relatively great discrepancies are surprising especially among the chemists.

[20] The deeper color of agar as compared with the usual color of broth makes necessary the use of some orange in this scale.

of its varying acid constituents. In the last 10 or 15 years phenolphthalein has been largely employed, yet it cannot be relied upon in every instance.

Park and Williams[21] state:

> Different indicators differ not only in delicacy but in the substances to which they react. A medium alkaline to litmus is acid to phenolphthalein showing that there are present substances possessing a character which litmus does not detect, weak organic acids and organic compounds, theoretically amphoteric but in which an acid character predominates.
>
> Thus a liter of bouillon becomes, on the addition of 1 per cent of peptone, more alkaline to litmus but decidedly more acid to phenolphthalein; 1000 cc. of water with 1 per cent peptone is acid to phenolphthalein to such an extent that 3.5 cc. of deci-normal NaOH is required to neutralize it. To litmus it is alkaline and requires 3.4 cc. of deci-normal HCl. Two per cent peptone doubles the difference. The same figures hold approximately true for peptone broth.

Eyre (1915) states that although meat infusion is always acid to phenolphthalein it may react neutral or even alkaline to litmus; again, if rendered exactly neutral to litmus, it still reacts acid to phenolphthalein; that this is due to the facts:

> (1) Litmus is insensitive to weak organic acids whose presence is readily indicated by phenolphthalein.
>
> (2) Dibasic sodium phosphate which is formed during process of neutralization is a salt which reacts alkaline to litmus but neutral to phenolphthalein.

On the other hand, MacNeal (1914) considers litmus the more useful:

> The neutral point indicated by litmus is very nearly the actual point in respect to acidity and alkalinity, and this point is not appreciably displaced in either direction by the addition of a neutral mixture of a feebly dissociated acid and its salts to the solution. The end reaction indicated by phenolphthalein when it turns pink is actually a point at which there is a slight excess of alkali. This is so nearly the neutral point in inorganic solutions, when electrolytic dissociation

[21] Third and fourth editions—1908 and 1910.

is marked, that the error is not appreciable. In solutions of organic substances, especially when considerable amounts of feebly dissociated substances such as are contained in peptone or gelatin, are present, this error becomes very appreciable. The discrepancy between the end point for litmus and for phenolphthalein will vary for different lots of media.

Naturally those media which contain litmus as an indicator to show acid production by the growth of bacteria, must be alkaline to litmus yet not too alkaline or the indicator is rendered useless. The testing of such media by the use of litmus paper is an unsatisfactory and crude method useful for only the roughest work. The use of a litmus solution (Merck's purified in 5 per cent aqueous solution) is far more satisfactory.

"Neutral to litmus" is "so and so" acid to phenolphthalein, the figure given varying with the writer. Muir and Ritchie place it at + 2.5, Stitt at + 1.5 boiling titration and about + 0.7 with the cold titration. Abbott gives + 2.5, Abel + 1.5 to 2.5 and Heinemann + 2, all depending on the shade of pink considered by the worker as suitable and the length of time the sample is boiled in the casserole.

In the modified method used in our laboratory, the figure is as low as + 0.6 or + 0.7 with media prepared with 1 per cent peptone. (This figure rises to + 1 when the boiling titration is used.) A 1 per cent peptone (Witte) solution in water is about + 0.2 to + 0.3 with phenolphthalein at room temperature and about + 0.4 at boiling figure.

It has been shown by Hildebrand (1913) and others (Clark, 1915; Bovie, 1915) that an indicator does not indicate the point of actual neutrality but merely a definite degree of hydrogen ion concentration. Where these two points coincide, i.e., where the hydrogen ion concentration at which the indicator changes color, is within the zone of absolute neutrality for a particular mixture of substances, this change of color in an indicator will, of course, be of significance. It would therefore seem desirable to select a specific indicator for each class of media. This could be accomplished only by determining the effect of added

alkali upon the electrical conductivity of the particular medium. Such measurement should be made by means of the hydrogen electrode. The measurements of potential are usually expressed in terms of hydrogen ion concentration, that is, acidity.

For an apparatus as described by Bovie (1915) the details of manipulation are as follows: A mixture of the medium with water in the same proportions as used for ordinary titrations is placed in a beaker kept at 30°C., and the precaution of excluding $CO_2$ observed. The indicator is added and the standard electrodes are immersed. Successive portions of decinormal sodium hydroxid solution are then added. After each addition of the alkali the potential of the mixture is observed and recorded.

The point at which the indicator gives its first change of color is marked. The additions of alkali should be continued and the potentials further noted until the curve changes its shape—that is, from concave to convex or vice versa. The actual point at which this change takes place is known as the point of inflection and is a true neutral point. The nearness of the indicator's point of change to this point of inflection determines its suitability for this particular class of media. The indicator in which the change comes nearest to this point should be selected for practical use. Work in choosing such indicators according to this method is planned.

When titrations are performed under ordinary circumstances at the boiling point, it is noticeable that making a decision as to the correct end point to phenolphthalein is much more difficult than when the temperature of 20° to 30° C. is used. This is due to the changes of ionization in the mixture caused by this considerable change in temperature.

Besides this, the constant presence of colloidal substances[22] in peptones,[23] phosphates and sugars in all media gives rise to

[22] Hildebrand (1913), Clark (1915) and others have shown that colloidal substances affect the sharpness of indicator end points.

[23] In the titration of the peptone solutions it was very difficult to get a sharp end point even at 20°C. The indicator (phenolphthalein) seemed to "flare" suddenly from the colorless state to a deeper pink than the one showing a really delicate end point.

a further depression of ionization, especially when these substances are decomposed by boiling.

The meagre results from the few investigations conducted in this field lead us to suspect that the presence of sugars in media may have a considerable influence on the effective acidity, that is, the dissociated acid principles (Hildebrand 1913). Work in these lines is to be continued.

## SUMMARY

Marked and continued hydrolysis, resulting in the formation of acid principles, occurred on successive heatings of meat infusions in the autoclave. The state of complete hydrolysis, i.e., the point at which no further acidity is produced, was not reached with these meat infusions (which had been subjected previously to boiling over the open flame for one to two hours) even after prolonged autoclaving at fifteen pounds pressure for eight[24] hours.

Hydrolysis did not occur in solutions of Liebig's beef extract subjected to similar heatings. In the portion which had been adjusted to the neutral point, however, slight hydrolysis did take place.

Those portions of meat infusion in which the natural acidity had been adjusted with normal sodium hydroxid to plus one (phenolphthalein) showed that hydrolysis occurred on application of heat and continued to do so after successive adjustments and autoclavings. There was produced in these portions as much acidity, approximately, as was developed on heating the corresponding uncorrected portions.

The further production of acidity after the addition of sodium hydroxid is due to the hydrolytic effect of heat in the presence of water, upon these portions of the medium unbound by alkali.

The fact that hydrolysis is promoted by heat makes inaccurate the estimation of acid ions in a batch of medium when there is

[24] With three of the meat infusions the time was extended from eight to fourteen hours.

taken as an index a sample[25] titrated at the boiling point with phenolphthalein as the indicator. Boiling has also a marked effect on the ionic concentration in media mixtures, hence the boiling titration called for in the Standard Method and followed more or less closely by many workers and recommended in the various text books, is subject to greater error than titrations performed at a temperature of 20° to 30° C.

In the adjustment of culture media not only is the desired end point more closely approximated by titration at 20° to 30° C. (see chart 6), but the misleading information of the boiling titration is avoided (see chart 4). Although according to Eyre (see page 211) certain acids are detected only at the boiling point, the *actual* reaction of media at the temperatures at which they are *used* is the object of vital importance. The conditions prevailing at these temperatures (incubator, 37.5°C., for some organisms and room temperature for others) are more closely indicated by titrations conducted at room temperature.

As shown in chart 4 the actual reaction of a medium titrated by the boiling method is really from 0.5 to 1 per cent lower than is indicated by the boiling titration figures.

The tests made on the reaction towards phenolphthalein of the various peptones on the market showed them to differ greatly in acidity. The effect on peptones of prolonged heating in tests similar to those on the meat infusions showed that hydrolysis occurred on the application of heat. The development of acidity took place after successive adjustments of reaction with sodium hydroxid, as with the meat infusions and the total amount of hydrolysis approximated closely that of the uncorrected portions. Here, too, prolonged heating of fourteen hours in the autoclave did not give complete hydrolysis.

In the usual titration methods, no one indicator gives all the

[25] That is, if the titration is performed after a preliminary boiling of the whole batch of meat infusion. When a titration is made on meat juice pressed out in the cold and containing added peptone dissolved at a low temperature, the boiling of the sample in the casserole is necessary to approximate future conditions after the boiling of the whole batch.

evidence desired in every case, e.g., the differences between litmus and phenolphthalein.

The necessary dependence on the change in color of an indicator to show the reaction of a solution, especially in the case of culture media, gives rise to error. This is due partly to the depressing effect of colloids, phosphates and some sugars which affect the sensitiveness of indicators; and also to variations of judgment with different workers as to the correct shade of color for an end point. Where it is desirable to avoid these influences titrations may be made by measurement of the electrical potential.

## REFERENCES


ABEL, RUDOLPH. 1912. Laboratory Handbook of Bacteriology, 2d ed. (translated by M. H. Gordon), p. 20.

ABBOTT, A. C. 1915. Principles of Bacteriology, 9th ed., p. 109.

BOVIE, W. T. 1915. A direct reading potentiometer. Jour. of Med. Research, *33*, 295.

CLARK, W. M. 1915. Reaction of bacteriologic culture media. Jour. of Infect. Dis., *17*, 109.

COMMITTEE ON STANDARD METHODS OF WATER ANALYSIS. 1905. A. P. H. A. Jour. Inf. Dis., Suppl. No. 1, 106.

COMMITTEE ON STANDARD METHODS OF WATER ANALYSIS. 1913. A. P. H. A., p. 126.

EYRE. 1915. Elements of Bacteriological Technique, 2d ed., p. 149.

HEINEMANN, P. G. 1911. Laboratory Guide in Bacteriology, 2d ed., p. 19.

HILDEBRAND, J. H. 1913. Some applications of the hydrogen electrode in analysis, research and teaching. Jour. Am. Chem. Soc., *35*, 847.

HISS AND ZINSSER. 1914. Text-book of Bacteriology, 2d ed., p. 117.

JORDAN, EDWIN O. 1914. Text-book of General Bacteriology, 4th ed., p. 29.

MACNEAL, WARD J. 1914. Pathogenic Microorganisms, p. 85.

MALLORY AND WRIGHT. 1915. Pathological Technique, 6th ed., p. 83.

MUIR AND RITCHIE. 1913. Manual of Bacteriology, 4th ed., p. 33.

PARK AND WILLIAMS. 1914. Pathogenic Bacteria and Protozoa. 5th ed., p. 88.

STITT. 1913. Practical Bacteriology, 3d, ed., p. 18.

SWITHINBANK AND NEWMAN. 1903. Bacteriology of Milk, 1st ed., p. 32.

# A NEW SPECIES OF ALCOHOL FORMING BACTERIUM ISOLATED FROM THE INTERIOR OF STALKS OF SUGAR CANE INFESTED WITH THE CANE-BORER *DIATRAEA SACCHARALIS*

WM. L. OWEN

*Louisiana Sugar Experiment Station, Audubon Park, New Orleans, La.*

Among the injuries sustained by sugar cane from various insects, that resulting from infestation with the sugar cane moth borer, *Diatraea saccharalis*, is generally regarded in Louisiana as of greatest importance. This pest is very widely distributed throughout the sugar cane growing countries of the world. Holloway (1912) who has made a very extended study of the occurrence of this insect in Louisiana, reported an average infestation of the crop of 1911 to be about 38 per cent. This infestation varied in degree in different sections, ranging from 0– to 78 per cent. The nature of the injury wrought upon sugar cane by this parasite is manifold. Barber (1911) attributed a greatly impaired germinating power of the cane to the injury of its eyes from the burrows made by the borer. Borer infested cane is also stunted in growth, and is rendered less able to withstand high winds, while its value is further impaired by the secondary infection of its interior by various fungi.

Of these various injuries caused by borer infestations, the last named is perhaps the most serious. Barber (1911) found the decrease in the purity of such cane to amount to 5.6 per cent. On the basis of recoverable sucrose per acre of cane, the above deterioration would amount to over 1000 pounds of sucrose for every 25 tons of cane.

Van Dine (1912) in investigating the borer injury to sugar cane in Porto Rico, reports a decrease of 5.8 in the purity of the juice, resulting from infestation with this parasite.

Among the secondary injuries to borer infested cane induced by fungi, those resulting from the infection with the Red Rot disease, is of first importance in this State. Of this injury, as well as of the general nature of the disease and the fungus, Edgerton (1911) has made a very extended study. That this fungus plays a most important part in the deterioration of the juice of canes infested with the borer, may be judged from the following analyses in Table III of the publicationof the above author.

| CONDITION OF CANE | SUCROSE | GLUCOSE |
|---|---|---|
| | *per cent* | *per cent* |
| Sound | 10.50 | 2.30 |
| Borer cane | 11.40 | 1.90 |
| Borer cane with red rot | 7.80 | 3.03 |

As the greatest infestation of cane with the red rot disease occurs as a sequel to borer attacks the economic importance of the control of this insect is obviously very great.

It would appear that the burrows made by the cane borer might offer suitable surroundings for the development of many species of bacteria which would inevitably find their way into the interior of the cane stalks. With a view of determining the part played by bacteria in causing a deterioration of the juice of borer infested cane, the writer began a preliminary investigation of this subject in the fall of 1914. At that time a large number of borer infested canes were examined, and an attempt made to isolate the species of bacteria occurring therein. The canes were brought to the laboratory, cut into short sections, and the portion of the stalk surrounding the borer wounds, washed off in a 1:1000 bichloride of mercury solution. A sterile platinum loop was then inserted as far into the interior of the wound as possible, and the injured tissue transferred to tubes of sterile culture media. As it happened the media employed for this purpose contained 10 per cent of sucrose. After a short incubation period, an examination of these tubes showed that practically every one of them was undergoing a vigorous fermentation. The presence of yeast cells in the tubes was quite

naturally expected, but a microscopical examination showed that bacteria rather than yeasts were predominant. Transfers to sterile plates, and the isolation of the predominating bacterial species, resulted in obtaining a culture, which showed a marked ability to ferment sucrose solutions. The copious amount of gas given off, which was tested for $CO_2$ with positive results, and the odor of alcohol, suggested the capacity of the species to induce an alcoholic fermentation. When transferred to sterile glycerine bouillon, the species also induced a vigorous fermentation, indicating its possible relationship or identity with other species of bacteria described in the literature. Although several species of alcohol forming bacteria have been isolated, this property may be regarded as rare. Among the most prominent of these species the following may be mentioned. *B. Fitzianus* was isolated from a cold hay infusion by Fitz (1880) and later more closely studied, and named by Buchner. This species forms ethyl alcohol from glycerine. Frankland and Fox (1889) isolated from the solid excreta of sheep an alcohol forming species of bacteria to which they gave the name *B. ethaceticus*. This species forms ethyl alcohol and acetic acid from glycerine. According to the work of Friedlander (1911) *B. pneumoniae* has the power of forming ethyl alcohol and acetic acid in nutrient solutions containing sucrose. Kruis and Rayman (1895) isolated from sour yeast a lactic acid bacterium that forms ethyl alcohol as a by-product.

Other species of alcohol forming bacteria are Duclaux's (1895) *Amylobacter ethylicus* isolated from garden soil, *B. butylicus* isolated by Fitz (1884) and two species isolated from malt decoctions by Henneberg (1909). There seems to be much in common between the characteristics of these species and our Louisiana organism, yet there are sufficient differences clearly to differentiate the latter from the former.

The *characteristics* of the sugar cane bacterium are as follows:

*Morphological.* Short thick rods with rounded ends, the individual cells averaging 2.8 $\mu$ in length and 1.0 $\mu$ in breadth. The rods occur chiefly in pairs, are frequently single, never in chains. The cells stain readily by aqueous and alcoholic solu-

tions of aniline dyes, and are Gram positive. The rods are non-motile, non-flagellated, and do not form endospores.

*Physiological characteristics.* This species does not liquefy gelatine at all. Milk is rendered slightly acid, and gas is developed after an incubation of 24 hours at 35°C. The consistency of the milk is unchanged in three days. Nitrates are not reduced. A fairly good growth occurred in a Novy jar from which all of the air was exhausted by means of a vacuum pump, and with the bottom covered with pyrogallic acid solution. The species is therefore a facultative anaerobe.

The following sugars are fermented by this species:

| | SUCROSE | GLUCOSE | LEVULOSE | MANNITE | LACTOSE | GALACTOSE | RAFFINOSE | MALTOSE | GLYCERIN |
|---|---|---|---|---|---|---|---|---|---|
| Gas | + | + | + | + | + | + | + | + | + |
| Growth in closed arm | + | + | + | + | + | + | + | + | + |

*Cultural characteristics.* On plain agar the colonies are small, rounded, but slightly raised, with entire edges. The surface is smooth and moist, with an amorphous interior structure. On glycerin agar the colonies are round greyish white, with a more glistening surface, but otherwise similar to the colonies on the plain agar. The sub-surface colonies are surrounded by gas bubbles resulting from the fermentation of the glycerin.

On agar streaks the growth is exceedingly rapid. Inoculated tubes show a marked growth along the line of the needle after six hours' incubation at 35°C.

On potato the organism forms a dirty white echinulate growth, slightly raised, with a glistening lustre. The growth is of a butyrous consistency. In bouillon the growth is very rapid, and the solution quickly becomes cloudy throughout. No film is produced. In bouillon containing 2 per cent of sucrose a vigorous fermentation follows inoculation with this species. The medium is rendered acid, and the acidity on the third day gives an acidifying coefficient of 3.8.

In its morphological and physiological characteristics the species in question strikingly resembles other species previously

isolated. In the table given below and in that on page 240 will be found the most prominent distinguishing features.

| CHARACTERS | SUGAR CANE ORGANISM B. SACCHARALIS | BAC. FITZIANUS (FITZ) | BAC. ETHACETICUS (FRANKLAND) | BAC. ETHACETO SUCCINICUS | HENNEBERG BACILLI | |
|---|---|---|---|---|---|---|
| | | | | | 1 | 2 |
| Size..... | 2.8 $\mu$ long 1.0 $\mu$ broad | Very large; similar to *B. subtilis* | 1.5-5.1 $\mu$ L 0.8-1.0 $\mu$ B | 1.7-1.5 $\mu$ L 0.5-1.0 $\mu$ B | – | Small short rods |
| Spore formation.... | – | + | – | – | – | – |
| Flagella..... | – | + (Probable) | + (Motile) | + (Probable) | | + (Motile) (+) |

From the foregoing table of characteristics of the various species of alcohol forming bacteria, and from that on page 240, it will be noted that the species isolated from sugar cane, differs from the others in the following essential points.

| FROM BAC. FITZIANUS | FROM BAC. ETHACETICUS | FROM BAC. ETHACETO SUCCINICUS | FROM HENNEBERG BACILLI | |
|---|---|---|---|---|
| | | | 1 | 2 |
| In smaller size and absence of spores | Not liquefying gelatine; absence of motility | Growth on agar and gelatine; absence of motility | Non-liquefaction of gelatine; absence of motility | Absence of motility; strong fermentation of sucrose, glucose, and levulose |

The observed differences in the characteristics of this species, seem sufficient to constitute it as a new species. Owing to its prevalence in borer infested sugar cane, the name *Bacillus saccharalis* seems appropriate.

In order to determine the amount of alcohol formed by this species in the fermentation of glycerin, a solution was prepared according to the formula of Frankland and Fox (1889), which is as follows:

| | grams |
|---|---|
| Glycerin | 60 |
| Peptone | 2 |
| Calcium carbonate (precipitated) | 30 |

*Cultural characters*

| CHARACTERS | BAC. SACCHARALIS | BACILLUS FITZIANUS | BACILLUS ETHACETICUS (FRANKLAND) | BACILLUS ETHACETO SUCCINICUS | HENNEBERG BACILLI | |
|---|---|---|---|---|---|---|
| | | | | | 1 | 2 |
| Colonies on plain agar | Round white raised, glistening | – | Thin veil-like growth almost invisible | – | – | – |
| Colonies on gelatine | Small round white; no liquefaction | Brownish yellow. No liquefaction | Small white dots. Medium liquefied | Yellow. No liquefaction. Colonies thin spreading | – | – |
| Agar streak | White glistening growth. Confined to needle track | – | | – | | |
| Agar stab | Non-characteristic. Surface growth white. Line at puncture echinulate* | – | | Thick growth white surface | – | |
| Gelatine stab | Line of stab filiform. No liquefaction | — | Beaded appearance; rapid liquefaction | Thin non-characteristic growth | Gelatine liquefied | |
| Potato | Vigorous raised moist white growth | – | Dirty white shining growth covering entire surface | Thick vigorous spreading growth yellow | | |
| Bouillon | Cloudy white sediment deposited on bottom of tube | – | White pellicle; white sediment | – | – | – |

* Echinulate.

Dissolved in 2000 cc. of the following salt solution:

| | DISSOLVED IN 5000 cc. WATER grams |
|---|---|
| Potassium phosphate | 5 |
| Magnesium sulphate | 1.0 |
| Calcium chlorid | 0.5 |

The solution was divided into one liter portions sterilized by the intermittent method, and inoculated with a pure culture of *Bacillus saccharalis*. After an incubation period of two weeks, an alcohol determination was made. The liquor was evaporated down to about a third of the original volume, until the distillate gave only a faint reaction with iodoform. After repeated distillations, the specific gravity of the 50 cc. portion was found to be 0.99744 which corresponds to 1.707 per cent of alcohol by volume. By dehydrating a small portion with fused carbonate of potash a solution was obtained which distilled at 79° to 80°C. showing it to be ethyl alcohol.

The residue was tested for acids and acetic acid was found to be present, using the ethyl acetate test. A mannite solution made up according to the same formula as the glycerin solution, except that 3 per cent of mannite was substituted for an equal weight of glycerine, was next tried. It yielded 50 cc. of a distillate with a specific gravity of 0.99836 corresponding to an alcohol per cent of 0.55 by volume. The presence of acetic acid was also detected in the residue.

Thinking that the low yields of alcohol in the two cases was due to the small quantity of assimilable nitrogen in the solution, 3 per cent of glycerin was added to plain bouillon, and the flask sterilized and inoculated as before. In this case the solution yielded 50 cc. of distillate of a specific gravity of 0.9864, which corresponds to an alcohol per cent of 4.895, which was much higher than in the previous experiment. The higher yield in the latter case indicated that there was a lack of nitrogen in the solution previously used. Frankland and Fox in their experiment with *B. ethaceticus* obtained a yield of 11.41 grams of alcohol from 60 grams of glycerin. In the experiments of the above investigators it was found that *B. ethaceticus* formed

1.63 parts of alcohol to one part of acetic acid, from mannite, while from glycerin the ratio of alcohol and acid was 2.11 to 1. Although *B. saccharalis* also forms alcohol and acetic acid from mannite, the ratio in which these products are formed was not determined in our experiments.

## ACTION ON SUGAR CANE JUICE

*B. saccharalis* grows vigorously in cane juice, and apparently induces a strong fermentation of its sugars. In order to determine its effect upon the composition of this substance, sterilized cane juice was inoculated and the following results were obtained. To one flask 1 per cent $CaCo_3$ was added, in order to neutralize acids formed during fermentation.

| SAMPLE | TREATMENT | TOTAL SOLIDS | INVERT SUGAR | SUCROSE | | ACIDITY | ALCOHOL PER CENT VOLUME | PURITY |
|---|---|---|---|---|---|---|---|---|
| | | | | Single polarization | Clerget | | | |
| | | | | *per cent* | *per cent* | | | |
| I | Inoculated + 1 per cent $CaCO_3$ | 7.75 | 0.73 | 4.4 | 4.86 | 1.2 | 0.53 | 56 |
| II | Control + 1 per cent $CaCo_3$ | 12.73 | 3.7 | 5.5 | 6.91 | 1.2 | | 43 |
| III | Inoculated | 8.75 | 1.3 | 4.2 | 5.92 | 2.8 | 0.79 | 47 |
| IV | Control | 12.43 | 4.27 | 5.7 | 7.36 | 1.8 | | 45 |

It will be noted from the above table that while some of the sucrose of the juice is inverted by the organism, a larger quanity of reducing sugars is destroyed. This results in an apparent increase in the purity of the inoculated flasks over the controls.

The question of what rôle this species plays in growing cane, and what effect its presence exercises on the composition of the juice of such cane, led to some inoculation experiments being conducted in 1914 and 1915 in the fields of the Sugar Experiment Station. In the first series of experiments the cane was inoculated by means of a small cork borer, and a pipette. The inoculations were made in the following manner. Holes were made in the cane with the cork borer, and 5 cc. of a water sus-

pension of a 24 hour-agar streak of the organism, was then introduced. The controls were treated in a similar manner, except that 5 cc. of sterile water was used instead of a culture. The holes in the cane were then sealed with grafting wax. All of the canes slected for the experiment were first examined for borer infestion, and only the borer free canes were used. About twenty inoculations were made in the first experiment, the varieties D. 74 and D. 95 being selected for the purpose. The inoculations were made on the 12th of October, and the canes were analyzed about the first of December, allowing nearly two months for the bacteria to develop. When the canes were analyzed, they were split through lengthwise, and transfers made with a sterile platinum loop, from the inoculation wounds to sterile glycerine bouillon. In the majority of cases the *B. saccharalis* was recovered from the inoculated portion of the cane, showing that it had remained in a living condition within the cane. The analyses of the canes were so variable, that it was decided to repeat the experiment the following year, using a slightly different method of inoculation. In September of the following year two rows of D. 74 cane were inoculated. Instead of a water suspension of the organism, a three days'old culture grown on sterile mashed potato was used as the inoculating material, and a blackleg vaccine injector was employed for the inoculations. The analysis of the cane was made in November, thus allowing an incubation period of two months for the organism to carry on its activities within the cane. The results of the analyses again showed that there was no marked deterioration of the juice of the inoculated cane. The juice of the inoculated cane, it is true, showed an average purity of 64.3 as against 67.4 for the control, but there were as many cases where the purity of the inoculated cane was higher than its control, as where it was lower. In this experiment, just as in the previous one, the organism was recovered from the inoculated portion of the cane, showing that it had remained in a living condition during the entire period. It is possible that the variations in the composition of the juice from different canes, even though they may be of the same size and in the same

stool, may have accounted for the negative results of the inoculation experiments. It is likely, however, that *B. saccharalis* does not induce any marked deterioration of the juice of growing cane, and indeed the apparently negative results which indicated a higher purity in the inoculated canes is well within the range of possible results from the action of the species. We have seen in the experiment on the action of this species upon cane juice how an increase of the purity of the juice may result from the fermentation of the invert sugar. It seems very probable that a similar result might follow from the presence of the species in growing cane. The occurrence of *B. saccharalis* in borer infested cane, and its survival in the interior of cane artificially inoculated with it, suggests a certain ability on its part to protect itself against the defensive properties of the plant. Sugar cane, like all other plants, possesses protective enzymes which tend to prevent the invasion of its tissues with organisms and their development therein, once they succeed in gaining an entrance. Browne (1906) reports a distinctly germicidal property of freshly extracted cane juice. He says:

> The darkening of vegetable tissues on the exposure to the air has been explained by Bertrand, to be due to the action of an oxidizing enzyme upon various tannin bodies, all more or less related to the polyphenols, and the query naturally arises does cane juice itself exercise any germicidal properties in connection with the natural phenomenon of darkening. The conclusion which we have reached in investigating this point is that cane juice does acquire for a time such germicidal characteristics. Counting the bacteria in the expressed juice of the cane at regular periods usually shows for several hours a uniform decrease in numbers; with juice from sterilized canes on the other hand, the bacterial content increases from the very start.

Again the author referred to states, that

> The living plant therefore does appear to protect itself against the invasion of microscopic parasites by forming toxic products.

The relation between the germicidal power of cane juice and the enzymes it contains, is suggested in the following observation by Browne, viz.,

The test for oxydase and catalase in cane juice becomes very feeble after ten or twelve hours, and with the disappearance of enzymic power, the number of bacteria begins to undergo a sudden increase. But it is more especially within the body of the cane itself that this germicidal action is most evident, and this we might expect not only from the colloidal and adherent character of the enzymes which renders them resistant to expression, but from the facts of localization, etc.

From this we must conclude that *B. saccharalis* possesses certain defensive properties which enable it to develop in spite of exposure to the germicidal action of the enzymes within the interior of the sugar cane. The prevalence of this interesting species in the interior of borer infested cane, and its predominance therein, further emphasizes the ability of *B. saccharalis* to overcome the defensive properties of the plant.

## REFERENCES

BARBER, T. C. 1911. Damage to sugar cane in Louisiana by the sugar cane borer. Bureau of Entomology Circular No. 139.

BROWNE, C. A. 1906. Fermentation of sugar cane products. Journal of American Chemical Society, *28*, No. 4.

DUCLAUX, E. 1895. Sur la nutrition intercellulaire. Ann. Inst. Pasteur *9*.

EDGERTON, C. W. 1911. La. Exp. Station Bulletin No. 133.

FITZ. 1880. Uber Schizomyceten Garungen. Ber. d. Deutsch. Chem. Gesellsch. *9*, *17*.

FRANKLAND, P. F. AND FOX, J. J. 1889. On a pure fermentation of mannite and glycerine (B. ethaceticus). Proc. of the Royal Society, London, *46*.

FRIEDLANDER. 1911. Jorgensen. Microörganisms and fermentation, 134.

HENNEBERG, W. 1909. Garungsbakteriologische Praktikum, 594.

HOLLOWAY, T. E. 1912. Field observations on sugar cane insects in the United States Bureau of Entomology Circular No. 171.

KRUIS, K. AND RAYMAN, B. 1895. Chemische Biologische Studien, 11 Bulletin internal Acad. Science de l'Emp. François Joseph. Prague.

VAN DINE, D. L. 1912. Damage to sugar cane juice by the moth stalk borer. Experiment Station of the Sugar Producers Association of Porto Rico. Circular No. 1.

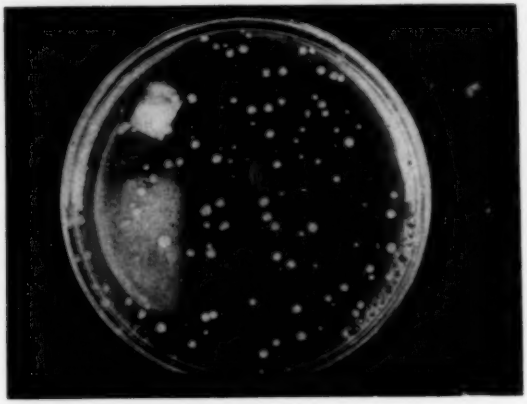


Fig. 2. A twenty-four hour growth of *B. saccharalis* on plain agar.

FIG. 1. A photomicrograph from a twenty-four hour agar growth of *B. saccharalis*.

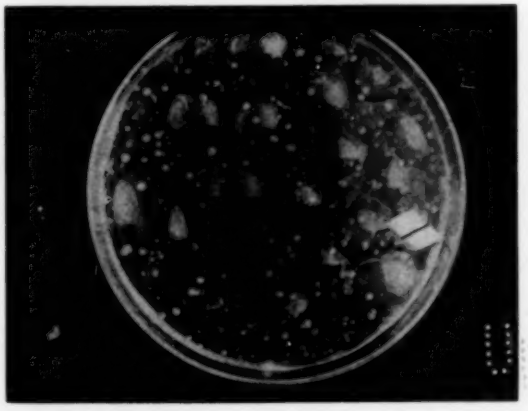


Fig. 3. A twenty-four hour growth of *B. saccharalis* on glycerin agar.

# ABSTRACTS OF AMERICAN BACTERIOLOGICAL LITERATURE

## ANIMAL PATHOLOGY

*The Maintenance of Virulence of Bacillus abortivus equinus.* E. S. Good and W. V. Smith. Jour. Med. Res., 1916, *33*, 493–498.

The authors present a note on the ability of the above bacillus to retain its virulence when kept under artificial cultivation over a long period. Inoculation of 1 cc. of a mixture of eight strains, representing only one-fifth of an agar slant, produced typical abortion in a mare protected with 200 cc. of hyperimmune serum. The strains used in this experiment had been isolated for periods varying from three to five years.—H. W. L.

*Studies to Diagnose a Fatal Disease of Cattle in the Mountainous Regions of California.* K. F. Meyer. Jour. Am. Vet. Med. Assoc., 1916, *48*, 552–560.

Discussion of the subject is divided into symptomatology, anatomical findings, bacteriological examinations and epidemiology.

Pieces of organs forwarded to the laboratory were subjected to microscopic examination without revealing bipolar organisms that could be regarded as *Bacterium bovisepticum* nor did ordinary culture methods give satisfactory results. Of about twenty-five rabbits inoculated with emulsions of liver infarcts, lymph nodes and spleen material, two died from a typical bipolar infection. The cultures isolated from these rabbits gave all the cultural identity reactions recognized as typical for *Bacterium bovisepticum*. The pathogenicity tests were characteristic except that large doses were necessary to produce fatal results. A three weeks old calf succumbed 22 hours after the intravenous injection of 3 cc. of a 20 hour old broth culture. Both strains were identical and serologically protected against each other and against strains of *Bacterium bovisepticum* from various sources in the United States.

Inoculation of guinea pigs with liver and infarct material caused death from infections with an undetermined anaerobe, *Bacillus coli* and diplococci. The anaerobe was not pathogenic to calves. Most of the mice inoculated with similar material remained alive or succumbed to the same anaerobe as did the guinea pigs.

The writer does not feel that the evidence thus far collected is sufficient to make a conclusive diagnosis of hemorrhagic septicemia but as a working hypothesis has assumed that the disease in all probability is hemorrhagic septicemia. The difficulties experienced brought forward again the fact that the bacteriological diagnosis of hemorrhagic septicemia is not as easy a procedure as is generally considered.—A. R. W.

*Vaccination Experiments Against Anthrax.* A. EICHHORN. Jour. Am. Vet. Med. Assoc., 1916, *48*, 669–686.

The writer reviews the development of measures for protecting animals from anthrax by such means as Pasteur's vaccination, spore vaccines, and injection of a serum as prepared by Sobernheim. The latter showed that the injection of an immune animal with increasing amounts of virulent virus would produce a serum possessing great protective value against anthrax. The author draws the following conclusions from his work with spore vaccines and serum:

1. Horses are suitable for the production of highly potent anthrax serum. Serum of such horses should protect large animals in 10 cc. doses.

2. The use of the serum treatment alone is indicated in cases where the infection has already occurred in a herd. Since the serum confers only a passive immunity it is advisable to revaccinate the herd in from three to five weeks by the simultaneous method.

3. The serum possesses great curative value. Depending on the severity of the infection, the curative dose is from 30 to 100 cc.; the injection may be repeated if necessary.

4. For the simultaneous treatment, a spore vaccine carefully standardized, is preferable to the ordinary Pasteur vaccine.

5. Spore vaccine should be employed in preference to the Pasteur vaccines for immunization with vaccine alone. The possibility of more accurate dosing of the spore vaccine and the better keeping qualities of the same, give this product a decided advantage over the other.

6. Experiments with concentrated serum and dry spore vaccine are very promising. This method would greatly simplify the vaccination process and also insure the product against subsequent contamination and deterioration.—A. R. W.

## BACTERIOLOGY OF WATER AND SEWAGE

*The Fundamental Principles of the Activated Sludge Process of Sewage Treatment.* T. CHALKLEY HALTON. Indiana San. and W. S. Assn., 1916, Eng. Contrg. *45*, 235–236.

The activated sludge process depends on the presence of biological life in the sludge under aerobic conditions.—L. P.

*Sanitary Features of Los Angeles Aqueduct.* B. A. HEINLEY. Mun. J., 1916, *40*, 35–37.

The water is brought 233 miles from Owens River to Los Angeles. The density of the population in the Watershed is 1.4 per square mile. In addition to the time in the aqueduct, reservoirs increase the normal storage period of 65 days under present conditions to 468 days. *B. coli* noted were traced to ducks. The mineral content ranged from 15 to 22 grains per gallon. Algae growths occasionally cause odors and taste, despite covered reservoirs on distribution system.—L. P.

*The Activated Sludge Process of Sewage Treatment.* G. J. FOWLER. Can. Eng. 1916, *30*, 227–228.

The author sketches the historical development of the process, and dwells on the "M7" process of adding bacterial cultures in the presence of iron in solution. Activated Sludge has 3 general effects (1) a clotting or clarifying action (2) a rapid oxidation of carbon and (3) nitrification. Much research is still required.—L. P.

*Hartford (Conn.) Waterworks Notes.* C. M. SAVILLE. Report Bd. Water Commrs., 1915, Mun. J., 1916, *40*, 333–334.

On account of the proximity of highways to reservoirs the water is sterilized before delivery. The raw water shows bacterial counts as high as 39,000 per cubic centimeter with *B. coli* found from 9 to 23 times in 10 cc. or less, every month. Treatment with 0.95 part per million available Cl has removed *B. coli* and 99.8 per cent of total bacteria. With $CaOCl_2$, 1 part per million available Cl was used or 25 pounds of bleach per million gallons. Liquid Cl used, 0.65 p.p.m. or 5.4 pounds per million gallons.—L. P.

*Vitality of the Cholera Vibrio in the Water of New York Bay.* A. J. GELARIE. Medical Record, 1916, *89*, 236.

The question whether the cholera vibrio dies or survives in native bay water is of importance in view of the fact that the waters about the Quarantine Station in New York may at any time be open to infection. Accordingly, a series of experiments was carried out to determine the viability of the cholera vibrio in native bay water.

Preliminary work demonstrated that the subjection of cholera vibrios to the osmotic pressure of bay water had no apparent effect.

Other organisms present in bay water were found to have an inhibitory influence upon the growth of the cholera vibrios. Vibrios not previously enriched with peptone were eliminated after 48 hours, those receiving preliminary enrichment after 7 to 47 days, the period varying according to the strain of cholera employed, the character of the water, and the quantity of bacteria added. Cholera vibrios added to sterilized bay water were found alive in some cases at the end of 285 days.

The demonstration of live vibrios in native bay water after a period of 7 to 47 days proves conclusively that every precaution should be taken to prevent pollution of bay waters.—M. W. C.

## IMMUNOLOGY

*Tuberculin Therapy.* HENRY L. SHIVELY. New York Med. Jour., 1916, *103*, 51.

General discussion of tuberculin therapy with report of three cases.—M. W. C.

*Experimental Study of the Effect of Emetinized Blood on the Typhoid Bacillus.* MARCUS BEEKMAN. Medical Record, 1916, *89*, 284.

The subcutaneous administration of emetine hydrochloride in 0.5 grain doses every six hours does not impart to the blood any bactericidal properties for the typhoid bacillus.—M. W. C.

*Newer Practical Points in the Treatment of Typhoid Fever.* BEVERLEY ROBINSON. Medical Record, 1916, *89*, 311.

In a discussion of the newer methods of treatment of typhoid fever, the advantages of vaccine treatment are considered as still questionable. —M. W. C.

*Note on a Skin Reaction in Pneumonia.* RICHARD WEIL. Jour. Exp. Med., 1915, *23*, 10–14.

The intradermic injection of pneumococcus autolysate in patients suffering from pneumonia produced such variations in the skin reactions that the author concludes that from a diagnostic standpoint, at least, the test has no significance.—B. W.

*The Preparation and Preservation of Complement.* LOYD THOMPSON. Jour. A. M. A., 1916, *66*, 652.

Fresh guinea pig complement is diluted 1:1 with an 8.1 per cent sodium chlorid solution. It is sealed in small tubes, 2 cc. to the tube. Before use 8 cc. of water is added to a tube, giving an isotonic 1:10 dilution of guinea pig serum. Complement held under these conditions is active for two weeks or longer.—G. H. S.

*Frontal and Maxillary Sinusitis and Sequelae. Due to Staphylococcus pyogenes albus.* RALPH OPDYKE. Medical Record, 1916, *89*, 18.

An account of a case in which a prolonged series of severe and obstinate involvements was found to be due solely to *Staphylococcus albus.* An autogenous vaccine, prepared and administered immediately after the beginning of the disease, was used without beneficial results.—M. W. C.

*Recent Developments in the Treatment of Leprosy.* VICTOR G. HEISER. New York Med. Jour., 1916, *103*, 289.

In reviewing the methods which have been used in the treatment of leprosy, the author states that vaccine treatment has apparently caused improvement in some cases, but in his own experience it has proved unreliable. In his opinion, the most satisfactory treatment consists of the subcutaneous administration of a mixture of chalumoogra oil, camphorated oil, and resorcin. The use of this mixture has caused cures in some cases, marked improvement in many. Examination of material taken from cured cases did not reveal the presence of leprosy bacilli.—M. W. C.

*Vaccine Therapy.* G. A. EHRET. Medical Record, 1916, *89*, 328.

Bacterins were used in a variety of infections—colon cystitis, bronchopneumonia, lobar pneumonia, chronic gonorrheal cystitis and prostatitis, gonorrheal arthritis, chronic articular rheumatism, neuritis, bronchial asthma, and otitis media—with successful results in every instance except one case of bronchial asthma. In the majority of cases, stock vaccines were used. The number of administrations and duration of treatment varied with the character of the case.—M. W. C.

*Immunological Studies in Pneumonia.* RICHARD WEIL and JOHN C. TORREY. Jour. Exp. Med., 1916, *23*, 1–10.

The authors injected guinea pigs subcutaneously with 4 cc. of the inactivated serum of pneumonia patients and from two to six days later tested the animals for hypersensitiveness by applying a pneumococcus autolysate to the excised uterus according to the method of Dale. The tests were controlled with serum from normal individuals or from patients suffering from diseases other than pneumonia. Of twenty cases of pneumococcus infection only two failed to produce sensitization, while in none of the control cases was a positive reaction obtained. The sensitizing antibody is present in the blood early in the disease and is found rarely after crisis. From the results it would appear that while the reaction is specific for the genus it cannot be used for group differentiation.—B. W.

*Progress in the Treatment of Skin Diseases.* G. M. MACKEE. New York Med. Jour., 1916, *103*, 441–444.

An interesting summary is given of the progress made during the last few years in the treatment of skin diseases.

Most important advances have been made in work upon the etiology of many of the dermatoses, particularly eczema.

The results reported with vaccine treatment are not uniform. Vaccine therapy has met with success in ringworm of the scalp, and in some cases, though not generally, in acne vulgaris. Bazin's disease, known as erythema induratum, and lupus vulgaris have been aided by tuberculin therapy.

Tuberculin is of no service, however, in the tuberculides nor in lupus erythematosus.—M. W. C.

*The Treatment of Typhoid Fever with Bacterins.* EDWARD WAITZFELDER. New York Med. Jour., 1916, *103*, 407.

Of sixteen cases of typhoid fever fourteen were treated with bacterins while two were used as controls and treated symptomatically. The bacterins used were prepared by the New York City board of health and were given intramuscularly in doses varying from 66 to 100 millions. It was found that the larger doses were the more effective.

The treatment was successful in that in the bacterin treated cases there was less fever, cardiac weakness, delirium, and exhaustion than

in the control cases. The period of convalescence as well as the period of acute illness was shortened, and in no cases were there any untoward results.—M. W. C.

*Treatment of Rheumatic Fever.* BEVERLEY ROBINSON. Medical Record, 1916, *89*, 11.

In discussing methods of treatment of rheumatic fever, the author mentions the use of vaccines and serums. Serums have proved to be without success and the advantages of vaccine treatment are still questionable. Whenever tried, vaccines should be used with great caution. Polyvalent vaccines should not be administered, as there is too great a risk of overburdening the system with non-specific antibodies. A further difficulty in the way of vaccine treatment is the fact that in the acute stage of the disease, the only time when bacteria can be isolated from the joints, vaccines do the least amount of good and their use is accompanied by greater local and general reactions than at a later period.—M. W. C.

*Antiblastic Immunity.* A. R. DOCHEZ and O. T. AVERY. Jour. Exp. Med., 1916, *23*, 61–68.

Ehrlich's side-chain theory, comprehensive as it is, fails to account for certain phenomena observed in immunological studies. Its author postulated a "third factor" to cover this decrepancy. Dochez and Avery now find that antipneumococcus serum possesses the power not only of inhibiting for a certain period the multiplication of pneumococci but also of inhibiting in varying degree their proteolytic and glycolytic functions. This power is present to a limited extent in the sera of certain normal animals, and, in human serum during the course of lobar pneumonia it appears or increases markedly at the critical period of the disease. The hypothesis that this retardation of bacterial growth is dependent upon the inhibition of metabolic function due to the presence of anti-enzymotic substances in antipneumococcus serum offers a possible explanation of the so-called "third factor" as well as a promising suggestion for further investigation.—B. W.

*The Complement Fixation Reactions of the Bordet-Gengou Bacillus.* M. P. OLMSTEAD and O. R. POVITZKY. Jour. Med. Res., 1916, *33*, 379–392.

Testing fourteen typical and four atypical strains of *Bacillus pertussis*, and nine strains of strictly hemoglobinophilic bacilli, by means of complement fixation, the authors report further confirmatory evidence of the individuality of *B. pertussis*, particularly their ability to differentiate between it and *Bacillus influenzae*. No differences in ability to bind complement were observed among twelve typical pertussis strains. Some cross reaction, although weak, was observed in two atypical strains and two strains of hemoglobinophilic bacilli.

The work was done with immune sera produced by the inoculation of rabbits with live cultures of the various organisms. The original

Wassermann technic reduced to one-tenth volume was used. The antigen which was found to give the best results was prepared as follows: A forty-eight hour growth on Bordet-Gengou medium was taken up in neutral distilled water and shaken for three to four hours in an electric shaker, the resulting emulsion allowed to stand at 56°C. over night, filtered through a Berkefeld, and the supernatant fluid used after being rendered isotonic with 9 per cent salt salution.—H. W. L.

*Pollen Extracts and Vaccines in Hay Fever.* SOLOMON STROUSE and IRA FRANK. Journ. A. M A., 1916, *66*, 712–715.

That pollen is the etiologic agent in hay fever cannot be questioned, but that it is the only factor is not certain.

It is possible that hay fever is a pollenosis associated with bacterial subinfection. It may be that the inhalation of pollen in susceptible individuals irritates the nasal mucosa rendering it more liable to bacterial infection and that this infection in turn favors the absorption of more pollen.

Cultures from the nose yielded in most instances pure cultures of *Staphylococcus albus*, although the pneumococcus and *Micrococcus catarrhalis* were occasionally found. Autogenous bacterial vaccines were prepared from the organisms isolated. Thirteen patients were treated with the bacterial vaccines; of these, 64 per cent showed signs of improvement.

A series of patients treated prophylactically with pollen extract alone showed decided improvement. The administration of vaccines following a previous pollen treatment resulted in seasonal cures. G. H. S.

*Equilibrium in the Combination and the Dissociation of Precipitates.* RICHARD WEIL. Proc. N. Y. Pathol. Soc., 1915, *15*, 132–134.

If a serum or other similar antigen be mixed with its specific precipitating anti-serum, the resulting precipitate never exhausts completely either of these two factors. Furthermore the serum of immunized animals sometimes contains both precipitin and precipitinogen. When a chemically pure antigen, namely crystallized egg albumen, is mixed with its specific anti-serum, a precipitate forms. The supernatant liquid can always be shown to contain one of the two factors, either egg albumen or antibody, but never both at the same time. Therefore it is concluded that under proper experimental conditions the precipitation reaction goes on to complete exhaustion of one factor and that equilibrium, in the sense of mass action, does not exist. The results of earlier observations are therefore explained by the presence of a multiplicity of antigens and antibodies, as was first suggested by Von Dungern. Furthermore the presence of a third colloid, such as rabbit serum, does not interfere with the completeness of the reaction.

In the subsequent discussion Dr. Weil stated that by heating a precipitin to 72° it is possible to deprive it completely of its precipitating property while the sensitizing value is retained almost unimpaired.—W. J. M.

## LABORATORY TECHNIQUE

*An Electrical Furnace for Sterilizing Inoculating Loops.* H. J. Corper. Journ. A. M. A., 1916, *66*, 187.

The author describes the construction of an electrical furnace for sterilizing platinum loops.—G. H. S.

*Two Laboratory Suggestions.* Geo. B. Lake. Medical Record, 1916, *89*, 422–423.

An eye shade for microscopical work is recommended.

By the addition of a small quantity of acid or alkali, tone may be restored to Wright's stain, which has deteriorated with age.—M. W. C.

*A Method of Obtaining Suspensions of Living Cells from the Fixed Tissues, and for the Plating Out of Individual Cells.* Peyton Rous and F. S. Jones. Proc. Soc. Biol. and Med., 1916, *13*, 73.

Bits of tissue are cultivated in plasma and the growing cultures flooded with trypsin dissolved in Locke's solution. The fibrin network is dissolved and the spherical living cells released. These are washed and plated anew.—W. J. M.

*A Simple Method for Blood Cultures.* Paul G. Weston. Jour. A. M. A., 1916, *66*, 507.

An ordinary vaccine ampule is half filled with culture medium. The neck is drawn to a capillary tube. A vacuum is obtained in the ampule and the capillary tube is sealed. A rubber tube, with needle for insertion into the vein, is placed over the capillary tube. The apparatus is then sterilized.

After puncture of the vein the capillary tube is broken. After the collection of blood no sealing is necessary as a firm clot plugs the needle.—G. H. S.

*A Stain for Tubercle Bacilli.* Emanuel Klein. New York Med. Jour., 1916, *103*, 217.

The author suggests as a substitute for the usual carbol-fuchsin, acid alcohol, methylene blue stain for tubercle bacilli, the following:

(1) 3 per cent alcoholic solution of crystal violet.
(2) 1 per cent aqueous solution of ammonium carbonate.
(3) 10 per cent solution of nitric acid (C. P.).
(4) 95 per cent alcohol.
(5) Saturated alcoholic solution of Bismarck brown of which enough is added to water to make a tincture of iodine color.

(1) and (2) are mixed in proportion 1:3. This is placed upon smear, which has been fixed in the usual manner, and allowed to steam and cool three successive times. Excess stain is poured off, slide washed in tap water. (3) and (4) are added alternately with rinsing after each, until specimen is perfectly colorless. Without washing, (5) is added for three minutes. Slide is dried and examined. The chief advantage of this stain is the contrast obtained with tubercle bacilli stained violet upon a light brown background.—M. W. C.

## MEDICAL BACTERIOLOGY

*Present Views in Respect of Modes and Periods of Infection in Tuberculosis.* MAZYCK P. RAVENEL. Jour. A. M. A., 1916, *66*, 613.
A general review of the literature on the subject.—G. H. S.

*Influenza.* A. H. DOTY. Medical Record, 1916, *89*, 455–456.
A general discussion of influenza with special emphasis upon means of prevention. M. W. C.

*Chronic Tonsillitis.* LOUIS FISCHER. New York Med. Jour., 1916, *103*, 147.
Bacteriological examinations of the throats of cases of chronic tonsillitis have shown the presence of *Staphylococcus aureus*, and an occasional streptococcus, never the Klebs-Löffler bacillus.—M. W. C.

*Peritonitis Following Acute Ovaritis of Anginal Origin.* RUSSELL M. WILDER. Jour. A. M. A., 1916, *66*, 659.
In the authors opinion many cases of so-called primary peritonitis result from infection of the throat passing to the ovaries and finally causing peritonitis. In the author's case diplococci and streptococci were found.—G. H. S.

*Two Unusual Strains of Diphtheroid Bacilli.* RALPH R. MELLON. Medical Record, 1916, *89*, 240.
A preliminary note briefly describing the cultural and biological characteristics of two strains of diphtheroid bacilli, both of which are pathogenic for animals. One of the strains is of especial interest culturally because of a most marked pleomorphism.—M. W. C.

*Common Affections of the Eye.* S. D. RISLEY. New York Med. Jour., 1916, *103*, 145.
Bacteriological examinations of the discharges from a large number of cases of ophthalmia neonatorum indicate that the disease is not always due to the gonococcus, but frequently to a variety of other microorganisms. Gonococcus is present in from 50 to 65 per cent of the cases.—M. W. C.

*The Control of Diphtheria Epidemics.* W. D. STOVALL. Jour. A. M. A., 1916, *66*, 804–806.
The author reports an epidemic of diphtheria in which the Schick test was employed. The use of the skin reaction and throat swabs together with prophylactic administration of antitoxin where indicated presents a most satisfactory method of combating epidemics of diphtheria.—G. H. S.

*Removal of Tonsils and Adenoids in Diphtheria Carriers.* S. A. FRIEDBERG. Jour. A. M. A., 1916, *66*, 810.
Report of 6 cases of diphtheria carriers in which the condition could not be remedied by the local application of kaolin.

Removal of the tonsils and adenoid tissue resulted in the prompt disappearance of the organisms upon culture.—G. H. S.

*A Study of the Etiology of Chronic Nephritis.* P. K. Brown and W. T. Cummins. Journ. A. M. A., 1916, *66*, 793–797.

From the study of a large number of cases of nephritis the authors conclude that venereal and other serious infections, chiefly streptococcus and pneumococcus, have a very definite bearing on the occurrence of advanced kidney disease.—G. H. S.

*Experimental Syphilis in the Rabbit Produced by the Brain Substance of the Living Paretic.* Udo J. Wile. Jour. Exp. Med., 1916, *23*, 199–202.

Brain tissue from living paretics easily produces experimental syphilis in rabbits and the spirochaetes contained in this living tissue constitute a virulent strain with a shorter period of incubation for the rabbit than exists with other strains.—B. W.

*The Incidence of Syphilis Among Juvenile Delinquents.* Thomas H. Harris. Journ. A. M. A., 1916, *66*, 102.

Wassermann tests performed on the sera of 365 juvenile delinquents taken without selection, gave positive results in about one-fifth of the cases. The author regards much of the infection as acquired rather than congenital. The relation of syphilitic infection to mental status is discussed.—G. H. S.

*Cultural Experiments with the Spirochaeta pallida Derived from the Paretic Brain.* Udo J. Wile and Paul Henry De Kriuf. Jour. A. M. A., 1916, *66*, 646.

Rabbit inoculation with paretic brain has yielded pure cultures of pallida. The strains may be cultivated in artificial media. The organisms from brain tissue are morphologically identical with spirochaetes derived from cutaneous syphilids, although the growth of the latter is much more luxuriant.—G. H. S.

*Diphtheria Carriers.* J. C. Geiger, Frank L. Kelly, and Violet M. Bathgate. Jour. A. M. A., 1916, *66*, 645.

Nose and throat cultures were taken from all contacts in six investigations. Nose cultures gave 42.2 in the percentage average of positives, throat cultures 7.9. Of all positive cultures 72 per cent were nose and 28 per cent throat.

The Schick test applied in one investigation proved of value in distinguishing between contacts and carriers.—G. H. S.

*Rôle of the Lymphatics in Ascending Renal Infection.* Daniel N. Eisendrath and Jacob V. Kahn. Jour. A. M. A., 1916, *66*, 561.

In a series of experiments on dogs and rabbits the authors have demonstrated that infection of the bladder with the *Bacillus coli*,

*Staphylococcus aureus* and *Proteus vulgaris* may result in infection of the kidney with these organisms.

Infection travels by way of the lymphatics in the wall of the ureter and not along the mucous membrane.—G. H. S.

*The Etiology of the Current Epidemic of Respiratory Infections in Chicago.* GEORGE MATHERS. Jour. A. M. A., 1916, *66*, 30.

Cultures obtained from the sputum, nasal discharge and the pharyngeal mucosa of twenty-four cases of respiratory infection revealed the fact that in seventeen instances the predominating organism was a hemolytic streptococcus, culturally resembling the *Streptococcus pyogenes* type. Pigment-producing streptococci and pneumococci were uniformly found. *B. influenzae* and *M. catarrhalis* were not present in any of the cultures.—G. H. S.

*The Cause of Rat-Bite Fever.* KENZO FUTAKI, ETSUMA TAKAKI, TENJI TANIGUCHI, and SHIMPACHI OSUMI. Jour. Exp. Med., 1916, *23*, 249–250.

A preliminary note in which is announced the finding of a spirochaete in the skin and in a lymph gland of patients suffering with rat-bite fever. The skin tissue and blood drawn from a patient when injected into monkeys, guinea pigs and white rats produced infection and the disease could be transmitted from these to other animals. Further details are promised.—B. W.

*The Etiology of Rat-Bite Fever.* FRANCIS G. BLAKE. Jour. Exp. Med., 1916, *23*, 39–60.

A case of rat-bite fever coming under the author's observation, terminating fatally and coming to autopsy, afforded an excellent opportunity for studying the etiology of this disease. An organism, which the author identifies as the *Streptothrix muris-ratti*, was demonstrated in a mitral vegetation and isolated in pure culture from the blood. The patient's serum contained strong agglutinins for the *Streptothrix*.—B. W.

*Influenza and Grippe in Infants and Children.* CARL G. LEO-WOLF. Medical Record, 1916, *89*, 226.

A discussion of influenza and grippe in children. All phases of the subject, such as history, etiology, pathology, treatment, etc., are treated in detail.

Emphasis is placed upon the fact that the two diseases are manifestly distinct entities. Both are due to bacterial infection, influenza being caused by the cocco-bacillus of Pfeiffer, grippe by one or more of a number of bacteria-pneumococcus, *Micrococcus catarrhalis*, bacillus of Friedländer, streptococci, and bacteria living as saprophytic parasites in the mouth.

In both influenza and grippe particular stress should be laid upon prophylaxis.—M. W. C.

*Rocky Mountain Spotted Fever.* HENRY C. MICHIE, JR. and HOUSTON H. PARSONS. Medical Record, 1916, *89*, 266.

A comprehensive investigation of Rocky Mountain spotted fever, which includes a résumé of all work done upon the disease as well as an account of the results of an extensive research on the fever as it occurs in the Bitter Root Valley, Montana. The report gives in detail the history, geographical distribution, etiology, symptoms, pathology, diagnosis, treatment, prophylaxis, prognosis, and epidemiology of the disease.—M. W. C.

*Remarks on B. Welchii in the Stools of Pellagrins.* W. H. HOLMES. Arch. Int. Med., 1916, *17*, 453–458.

In a study of the stools of pellagrins an abnormally large number of organisms of the *B. Welchii* group have been found. These organisms are able to produce diarrhea in the presence of a high carbohydrate diet, which can be cured by the substitution of a protein diet. Since he believes that pellagra is caused by a high carbohydrate diet, the writer suggests a further investigation of the rôle of *B. Welchii* in this disease.—G. H. R.

*The Treatment of Infections of Accessory Sinus.* A. M. MACWHINNIE. New York Med. Journ., 1916, *103*, 213.

Theoretically, the treatment of ethmoiditis should consist in the administration of an autogeneous vaccine made from all types of bacteria isolated. In most cases these vary in number from three to five. The use of such a vaccine has met with marvelous results in a few cases, but the average of successful treatments is 30 per cent.

The author recommends a system of cleansing to be used in conjunction with the application of his suction pump.—M. W. C.

*Salvarsan in Primary Syphilis.* ALEXANDER A. UHLE and WM. H. MACKINNEY. New York Med. Jour., 1916, *103*, 6.

Treatment of primary syphilis with salvarsan is most successful in cases in which a diagnosis is established sufficiently early to allow prompt administration of the drug. The earliest positive diagnosis of syphilis can be made by examination by dark field illumination of the expressed serum of a suspected sore. A Wassermann reaction is not positive until the seventh to fourteenth day after the appearance of the chancre. The Wassermann reaction is of value in the diagnosis of syphilis, as a positive reaction means the onset of systemic syphilis, while a negative reaction, for practical purposes, means a local infection only.—M. W. C.

*The Prompt Cure of Gonorrhea.* GEORGE A. WYETH. New York Med. Jour., 1916, *103*, 244.

Treatment with a 0.25 to 0.5 per cent solution of protargol, if begun within twenty-four hours after the appearance of a purulent discharge,

has resulted in a cure within five to seven days in 60 per cent of the author's cases of gonorrhea.

New, well developed cases, where no evidence of phagocytosis is shown, are more stubborn in yielding to treatment than cases where the gonococci are mostly intracellular. In the latter group of cases, the use of vaccines is indicated.—M. W. C.

*A Study of the Bacteriology of Chronic Prostatitis and Spermatocystitis.* HARRY B. CULVER. Jour. A. M. A., 1916, *66*, 553.

Review of literature. Detailed account of technic employed by the author.

34 cases examined organisms were recovered from 70 per cent. Twelve different organisms were isolated—*Staphylococcus albus*, *Streptococcus hemolyticus*, *Gonococcus*, diphtheroid bacillus, *Micrococcus tetragenus* and *M.catarrhalis*, *Bacillus proteus*, a colon-like bacillus, anaerobic staphylococcus and streptoccoccus and unidentified gram-negative cocci and diplococci.

Skin tests, agglutination and opsonic determinations showed that in 66 per cent of the cases tested the organisms isolated appeared to be specific for the infected individual. Vaccine treatment was apparently beneficial.—G. H. S.

*Gallbladder Diseases.* C. H. MAYO. New York Med. Jour., 1916, *103*, 433–436.

Diseases of the gallbladder are of infectious origin. Cultures made from the tissues of actively diseased gallbladders. and inoculated intravenously into experimental animals caused disease of the gallbladder, even to occasional stone formation, in 61 per cent of 41 animals. (Rosenow.)

Stones removed from the gallbladder may retain living bacteria for years. The stone is the result of the infection, not the cause of the disease.

The mode of infection is not yet known. Several theories have been advanced, the most probably being that of Rosenow—that the tissues of the gallbladder are open to infection through the vascular system. Typhoid bacteremia is frequently the etiological factor and in this case the attack is undoubtedly through the vascular system.

Several methods of treatment are described.—M. W. C.

*The Bacteriology of the Recent Grip Epidemic.* CHARLES HALPIN NAMMACK. Medical Record, 1916, *89*, 369.

Cultures made from 50 cases, which had been clinically diagnosed as grip, revealed the following findings:

Influenza-like bacilli in 19 cases, in 6 cases alone and in 13 associated with other microörganisms.

| | |
|---|---|
| **Pneumococcus** | **in 13 cases** |
| **Hemolytic streptococcus** | **in 6 cases** |
| **Friedlander's bacillus** | **in 3 cases** |
| **Staphylococcus** | **in 3 cases** |
| **Micrococcus catarrhalis** | **in 1 case** |

These organisms were isolated by means of sputum and nasal cultures.

Two cases are reported in which after recovery there were isolated pure cultures of capsulated pneumococci in one and *Bacillus influenzae* in the other. Such findings emphasize the necessity of taking precautions against infection from persons who are undoubtedly carriers of organisms that may cause grip.—M. W. C.

*So-Called Grippe.* J. B. RUCKER, JR. New York Med. Jour., 1916, *103*, 294.

Bacteriological examinations of 20 cases of so-called grippe presented findings as follows: 8 containing pneumococcus, typical at least in morphology; 6 containing atypical pneumococcus or *Streptococcus mucosus*; 20 containing small gram positive biscuit-shaped diplococci; 2 containing the bacillus of Pfeiffer, in smear only.

Of all organisms isolated, pathogenicity for animals was established only with the small gram positive diplococci. These organisms caused death of mice in 2 cases, and typical grippe-like symptoms in a rabbit in 1 case. Results of examination of these 20 cases suggest that the etiological factor in the recent epidemic of so-called grippe is the small gram positive diplococcus isolated from all cases.—M. W. C.

*Routine Wassermann Examinations of Four Thousand Hospital Patients.* I. C. WALKER and D. A. HALLER. Jour. A. M. A., 1916, *66*, 488.

Routine examinations of 4000 hospitals admissions were made. The prevalence of unsuspected syphilis and the frequency of positive reactions in various diseases were studied.

The reaction was positive in 600 cases. Of the 600 positive patients 48 were in the very early stages of syphilis, 306 were in a later stage, and 120 were in cases having involvement of the central nervous system. 13 cases were congenital.

There were 54 positive cases with aortic disease, 10 with epilepsy, 10 with disease of the liver, 10 with disease of the kidney, 9 with pneumonia, 7 with diabetes, and 13 distributed among miscellaneous diseases.

The authors conclude that syphilis is more prevalent than is ordinarily supposed and that infectious diseases, such as typhoid fever, pneumonia, tuberculosis and scarlet fever, do not cause false positive reactions.—G. H. S.

*The Treatment of Gastric Ulcer.* A. F. R. ANDRESEN. Medical Record, 1916, *89*, 457–459.

A rational, specific therapy of gastric ulcer should be based upon the fact that this pathological conditions is due to an infective process, the etiological agent of which is *Streptococcus viridans*.

Treatment in cases of simple ulcer should consist first of removal of the cause of infection, second of rest of the infected part, and third of efforts to overcome the infection and to repair the injured tissues.

Under the third heading, the use of autogenous vaccines is of chief importance. Such vaccines have proved of great assistance in clearing up foci of infection, as well as in exerting a beneficient action on ulcer symptoms. Vaccines were used by the author in 38 cases with constantly successful results.—M. W. C.

*The Treatment of Diphtheria Carriers with Iodized Phenol.* W. O. Ott and K. A. Roy. Jour. A. M. A., 1916, *66*, 800–802.

The treatment of 17 diphtheria carriers by the use of iodized phenol is reported.

The solution (60 per cent phenol, 20 per cent iodine crystals and 20 per cent glycerin) was swabbed over the tonsils, uvula and posterior wall of the pharynx in pharyngeal cases and over the entire anterior part of the nasal cavity in nasal cases. Applications were made every 48 hours until negative cultures were obtained.

No bad results were noted from the use of the preparation although the application is painful for half a minute or less until the anesthetic action of the phenol takes effect.

Negatives cultures were obtained in 35 per cent of the cases after one application; in 29 per cent after the second; in 12 per cent after the third; in 6 per cent after the fifth and in 12 per cent after the sixth. One case required 9 applications.

Fifteen cases were followed from one to three weeks after leaving the hospital and yielded negative cultures.—G. H. S.

*The Extent and Significance of Gonorrhea in a Reformatory for Women.* Elizabeth A. Sullivan and Edith R. Spaulding. Journ. A. M. A., 1916, *66*, 95.

An exhaustive study of 500 women delinquents with respect to the prevalence of gonorrheal infection; the nature, duration and extent of the infection, together with its susceptibility to treatment; the effect of the infection in producing other pathologic conditions; its relation to birthrate; and general considerations of an economic and sociologic nature.

Among 522 cases examined, 395 or 75.7 per cent were found to be gonorrheic. The average duration of infection was 4 years, 5 months, the case of longest duration being 26 years. In 82.7 per cent of the cases there had been no cessation of clinical symptoms since the initial infections. With respect to treatment during the course of the infection, the clinical history of 378 cases showed that but 1 per cent had received adequate medical treatment.

A comparison of the birthrate among gonorrheic and non-gonorrheic women showed that the average number of children among the former was 1.1, among the latter, 2.8.

The amount of treatment required to eradicate the clinical symptoms depends on the duration of the disease previous to treatment. After disease has lasted from 4 to 6 months without treatment, 10 to 12 months' treatment is usually necessary.—G. H. S.

*The Epidemiology of Tuberculosis.* F. C. SMITH. Journ. A. M. A., 1916, *66*, 77.

A general discussion of the subject.

The climate and topography of the country cannot of themselves constitute immune zones. Such areas are simply uninfected territory.

Infection of a majority of all persons occurs before the age of 12 years. Such factors as street dust, flies, water and fomites are probably of less moment in causing infection then direct contact. The lymph glands as avenues of infection are significant.

The importance of infection with the bovine type is indicated by the fact that 8 per cent of deaths from tuberculosis are due to this agent.

While it is granted that certain occupations predispose to tuberculosis and that age, social condition, economic state, and race may be potent factors, It is most certain that physical exhaustion, whatever may be its cause, entails the failure of some of the natural defenses, and latent infection becomes active.

Gross infection should be avoided; a diagnosis should be made at the earliest possible time, but in the eradication of tuberculosis the greatest problem is the economic one.—G. H. S.

*Tuberculosis.* HERMANN M. BIGGS. New York Med. Jour., 1916, *103*, 168.

In reviewing the progress made in the treatment and control of tuberculosis during the past 20 years, the author states that while much has been done toward eradicating the disease, researches upon tuberculosis have not added anything essentially new to the knowledge of the subject. A clearer definition has been given to certain phases of the disease, such as the establishment of the facts that bovine infections play practically no part in the production of pulmonary tuberculosis, but do cause 30–35 per cent of the tuberculosis of lymph nodes of children under five years; that pulmonary tuberculosis is practically always the result of the direct transmission of tubercle bacilli from the sick to the well; and that the disease is definitely preventable.

Neither a specific treatment nor an effective method of producing insusceptibility for tuberculosis has been discovered. Tuberculins and various forms of modified vaccines are receiving less recognition than formerly. The evidence of the wide dissemination of tuberculous infections in early life renders the use of tuberculin of little value as a diagnostic agent.

With the use of the X-ray, some progress has been made in diagnosis and with the aid of an earlier diagnosis a larger per cent of recoveries is probable.

As a constructive program for further eradication of tuberculosis, emphasis should be laid upon extensive improvements in preventive measures. Among these are disinfection of tuberculous material, caution in disseminating the disease, increased facilities for bacteriological diagnosis, adequate provision for institutional care of tuber-

culous cases, the extension of nursing service of the type now done by the visiting public health nurse, insistence upon pasteurization of milk supplies, and vigorous prosecution of the educational compaign.—M. W. C.

*The Period of Life at which Infection from Tuberculosis Occurs most Frequently.* S. ADOLPHUS KNOPF. Medical Record, 1916, *89*, 47.

A study of several still unsolved problems of tuberculosis brings forth the following facts, based upon the opinions and statistical evidences of a large number of authorities upon tuberculosis and children's diseases.

Tuberculous diseases in childhood, compared with tuberculous infection, is relatively rare (36 per cent). Tuberculous infection in infants and young children is exceedingly frequent and the majority of cases in the adult can be traced to a childhood infection. Such an infection is most apt to become active about the fifteenth year; if not then, between 18 and 30. A tuberculous infection contracted in later life usually occurs between the ages of 20 and 35. It is probable that prenatal infection is more frequent than has been generally believed. The frequency of infection increases with the age of the child and is affected by environment.

Lungs and lymph nodules are the organs most frequently involved in children; secondly, bones; thirdly, intestines; and fourthly, meninges.

The most common sources of infection are contact with tuberculous individuals and infected food; especially milk from tuberculous cows.

The most successful means of combating tuberculosis is to diminish the source of infection in childhood. In order to do this, there must be a radical change in our present regulations in regard to the disease and a much more extensive provision for the care of the infected. Particular attention should be given to preventive measures, especially the establishment of an extensive educational system and the improvement of social conditions. These changes can be best accomplished by a Federal Commission on Tuberculosis.—M. W. C.

*The Epidemic of Typhus Exanthematicus in the Balkans and in the Camps of Europe.* BERT. W. CALDWELL. Jour. A. M. A., 1916, *66*, 326.

A general discussion of the epidemic, its causes and extent; and the means employed in its control.

One person out of every five of the population was attacked by typhus, the fatal cases numbering 135,000. The hospital mortality ranged from 19 to 65 per cent. Conditions (cold weather and congestion of population) peculiarly favorable to the distribution of the disease obtained.

The body louse is a certain, and the head louse a probable, agent in its transmission. Evidence of any other mode of transmission is entirely lacking. With proper hygienic precautions non-immunes are practically safe from infection. The incubation period of the disease

is about fourteen days. Eruption follows the onset closely and reaches its maximum intensity on the fifth day. It is during this five-day period that there is greatest danger of infection. The disease seems to be a general septicemia, the on.y discovery relative to its pathology being the recovery from the spleen of an organism resembling the Plotz organism.

In the eradication of the epidemic the American Red Cross Sanitary Commission employed such measures as fumigation of all hospitals, barracks, schools and other foci of infection, bathing patients and sterilizing their clothing, maintaining quarantine of patients, and the institution of measures of general sanitation.

The treatment of typhus fever is unsatisfactory and is supportive and symtomatic in character. The serum prepared by Nicolle, or the vaccine prepared from the Plotz organisms tends to abort the disease and apparently has therapeutic value. The prophylactic value of the Plotz vaccine is problematic.—G. H. S.

## PROTOZOA AND OTHER ANIMAL PARASITES

*Trichinosis.* Arthur R. Elliott. Jour. A. M. A., 1916, *66*, 504.

A case of trichinosis is reported from which actively motile trichina larvae were found in the spinal fluid.—G. H. S.

*Filaria Sanguinis Hominis.* Codis Phipps. Journ. A. M. A., 1916, *66*, 266

The author reports a case of infection with *Filaria sanguinis hominis* (*Filaria nocturua*, *Filaria bancrofti*) in which a cure was effected by the administration of salvarsan.—G. H. S.

*Dermatitis Herpetiformis.* M. F. Engman and Robert Davis. Jour. A. M. A., 1916, *66*, 492.

It is probable that the endameba is an etiologic factor in a certain percentage of cases of dermatitis herpetiformis.

In such cases the administration of emetin hydrochloride has proved of value.—G. H. S.

*Trichiniasis.* Michael G. Wohl. Medical Record, 1916, *89*, 98.

A general review of the disease with the report of one case.

In discussing methods of treatment, the author states that the administration of vaccines prepared from trichinae derived from infected hogs would be a logical step, as specific antibodies have been demonstrated in the blood of patients suffering from trichiniasis.—M. W. C.

*Thionin as a Diagnostic Stain for Pyorrhea Alveolaris.* Martin Dupray. Jour. A. M. A., 1916, *66*, 507.

An excellent diagnostic stain for endamebae may be prepared as follows:

| | |
|---|---|
| **Thionin** | **0.5 gm.** |
| **Distilled water** | **100.0 cc.** |
| **Phenol crystals** | **2.0 gm.** |

The smear is air-dried, fixed in flame, stained for a few seconds while warm, washed and dried.

The cytoplasm of the endamabae stains a light purplish violet, the nuclei a deeper reddish violet. Ingested blood corpuscles are nearly black. Pus cells are a light blue. Bacteria are well stained, the fusiform bacilli and spirilla being especially plain.

The stain deteriorates in three to four months.—G. H. S.